



PHD

Assessing the Wine Yeast *Metschnikowia pulcherrima* for the Production of 2-Phenylethanol

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Assessing the Wine Yeast *Metschnikowia pulcherrima* for the Production of 2-Phenylethanol

TANAKORN CHANTASUBAN

A thesis submitted for the degree of Doctor of Philosophy

University of Bath

Department of Chemical Engineering

September 2016

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Abstract

2-phenylethanol (2PE) is a valuable fragrance compound which gives a rose-like aroma. As such 2PE is one of the highest used fragrances globally. While 2PE is predominantly produced from petrochemical resources, there is a growing market for a naturally derived alternative for food products. 2PE from natural sources is priced so highly due to limited supply from rose petals. Recently, a few reports have demonstrated the production in yeasts through both the *de novo* production from glucose and *ex novo* biosynthesis with L-phenylalanine as a precursor. While these are promising most of the yeasts used can only produce low titres under optimal conditions, and the fermentation still appears to be too expensive. In this investigation the wine yeast *M. pulcherrima* was selected to be assessed for 2PE production. *M. pulcherrima* is known to produce 2PE in small titres in wine production though has yet to be explored as a platform for this product. *M. pulcherrima* has several advantages as a yeast platform, in that it produces a range of antimicrobials which can ward off invasive species, allowing for less sterile control in any large scale fermentation.

M. pulcherrima was demonstrated to be able to produce 2PE in high titres in the batch mode through *de novo* synthesis of glucose, producing up to 1 g/L in shake flasks on the lab scale. Arabitol was also observed in the fermentation broth and was produced up to 20 g/L. The fermentation was then scaled up to 2L in batch mode. From these experiments, up to 700 mg/L of 2PE was produced. This is substantially more than any other yeast in the literature to date. Though when xylose or glycerol was present then 2PE production was severely limited. *M. pulcherrima* was also demonstrated to be able to produce 2PE by bioconversion from phenylalanine up to 1.5 g/L. This 2PE concentration is suggested to be threshold of toxicity to *M. pulcherrima* by the toxicity study. The production of 2PE could be increased substantially by introducing an absorbent into the process.

Liquid solvents and solid adsorbents were assessed to increase 2PE production, used as in-situ 2PE adsorbents. Oleyl alcohol was found to be a good solvent for in-situ extractive solvent in *M. pulcherrima* culture and increase the production to 3.3 g/L which is higher than 2PE tolerance threshold of the yeast. Activated carbon was also found to be an excellent 2PE adsorbent, with maximum Langmuir adsorption capacity up to 0.807 g/g. 2PE synthesis with activated carbon as an in-situ adsorbent can increase 2PE production to 14 g 2PE/L.

Finally, the process was scaled to 2L and run in batch, continuous and semi-continuous modes. This study demonstrates that not only is *M. pulcherrima* a viable organism to

produce 2PE but it has the potential to be scaled up and run in a more cost effective semi-continuous mode when coupled to a continuous extraction technique.

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List of Abbreviations

2PE	2-phenylethanol
A7.1	Unknown product compound from <i>M. pulcherrima</i>
ADH	Alcohol Dehydrogenase
ADP	Adenosine Diphosphate
ArDH	Arabitol Dehydrogenase
ATP	Adenosine Triphosphate
B7.8	Unknown product compound from <i>M. pulcherrima</i>
C/N ratio	Carbon/Nitrogen ratio
C _{10.6}	Unknown product compound from <i>M. pulcherrima</i> , (Arabitol)
CAD40	Sorption resin
CD180	Sorption resin
DAG	Diacylglycerol
DAHP	(3-Deoxy-o-arabiono-heptulosonate-7-phosphate
DDC	Dodecane
DM11	Sorption resin
DO	Dissolved Oxygen
E _{12.5}	Unknown product compound from <i>M. pulcherrima</i> , (Glycerol)
E4P	Erythrose 4-phosphate)
F _{14.4}	Unknown product compound from <i>M. pulcherrima</i> , (Acetic Acid)
F6P	Fructose 6-phosphate
FFA	Free Fatty Acid
G _{15.8}	Unknown product compound from <i>M. pulcherrima</i>
G6P	Glucose 6-phosphate
GAC	Granular Activated Carbon
GAC0412	Granular Activated Carbon (4-12 mesh)
GAC2040	Granular Activated Carbon (20-40 mesh)
GMO	Genetically Modified Organism
HDPE	High Density Polyethylene
HPLC-RI	High Pressure Liquid Chromatography with Refractive Index Detector
HPLC-UV	High Pressure Liquid Chromatography with Ultraviolet Detector
HRT	Hydraulic Retention Time
HZ801	Sorption resin
HZ818	Sorption resin
HZ841	Sorption resin
I _{17.1}	Unknown product compound from <i>M. pulcherrima</i>
J _{18.2}	Unknown product compound from <i>M. pulcherrima</i>
KDC	2-Keto-acid Decarboxylase
L-phe	L-phenylalanine
M	Molar
mM	millimolar
MSGJ	Modified Synthetic Grape Juice (medium)
MSGJ	Xylose Modified Grape Juice (medium)
N/C ratio	Nitrogen/Carbon ratio
NAD	Nicotinamide Adenine Dinucleotide
NAP	non-Aqueous Phase
OA	Oleyl Alcohol
OD	Optical Density

OD ₆₀₀	Optical Density at 600 nm
PA	Phosphatidic acid
PAC	Pow dered Activated Carbon
PEP	Phosphoenol pyruvate
PPG	Polyethelene glycol
PPP	Pentose Phosphate Pathw ay
rpm	round per minute (rotation)
RT	Retention Time
s.d.	Standard Errors
SBR	Styrene butadiene
SGJ	Synthetic Grape Juice (medium)
TAG	Triacylglycerol
TCA Cycle	Tricarboxylic Cycle
TPPB	Tw o-phase Partitioned Bioreactor
Tryp	Tryptophan
Tyr	Tyrosine
XAD1180	Sorption resin
XAD7HP	Sorption resin
XGJ	Xylose Grape Juice (medium)
YMS	Yeast Extract-Manitol-Sorbose (medium)
YPD	Yeast Extract-Peptone-Dextrose (medium)
ZSM-5	Zeolite Socony Mobil-5

Chapter 1

Introduction

1.1 *Metschnikowia pulcherrima*

Metschnikowia Pulcherrima is an ascomycetous yeast in genus *Metschnikowia*, which used to be grouped in the *Candida* clade but has been split into a separate genus *Metschnikowia*. (The scientific classification of the yeast is shown in Table 1.1.) The yeast is found generally in flowers, fruits, plants and insect vectors in many parts of the world. The yeast is commonly found growing on grapes used in the wine industry, and therefore plays a vital part in the early stage of wine fermentation. The products produced including aromatics and esters lend a lot of wines their unique aroma. It is well regarded in biological control for use in spoilage control in postharvest fruits and food and control in plant diseases. Its killer behaviour is currently believed to mainly involve iron depletion caused by releasing of chelating agents [1-3]. In addition, various enzyme activities were widely studied. Recently, it was popularly known for its lipid production which is similar to palm oil [4, 5].

Metschnikowia sp. are ascomycetous yeasts identified by the multilateral budding in the vegetative growth phase and the presence of needle-shaped ascospore (sexual spore) in elongate asci. The yeasts in the genus are separated in two groups according to the traits found in the yeast from different habitats which are the terrestrial habitat group, in which *M. pulcherrima* is placed in, and the aquatic habitat group. The former group is typically found in flowers, fruits and insect vectors and the latter is often parasitic in the aquatic invertebrates [6].

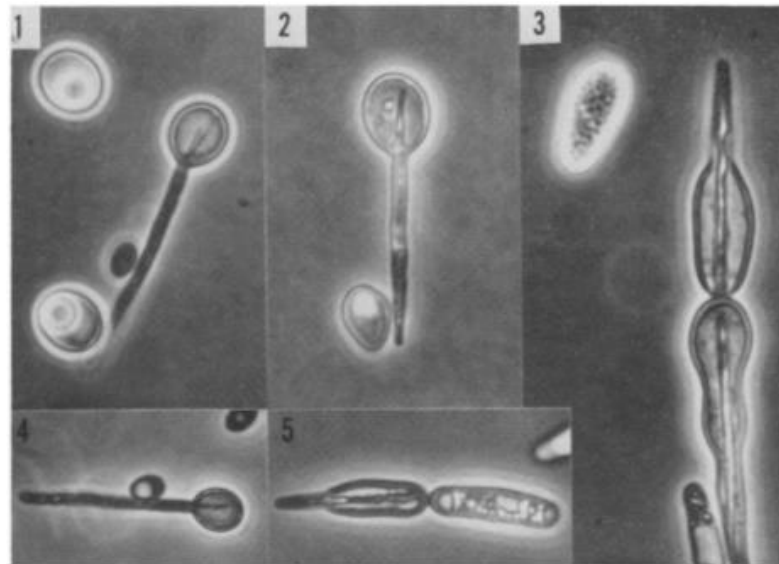


Figure 1.1 *Metschnikowia* sp. showing the long cylindrical needle-shaped asci, which figure 1, 2 and 4 are *Metschnikowia pulcherrima* (taken from [7])

Table 1.1 Scientific classification of *Metschnikowia pulcherrima*

Kingdom	Fungi	Synonyms
Phylum	Ascomycota	<ul style="list-style-type: none"> • <i>Candida pulcherrima</i> • <i>Torula pulcherrima</i> • <i>Torulopsis Candida pulcherrima</i> • <i>Rhodotorula pulcherrima</i> • <i>Saccharomyces pulcherrimus</i> • <i>Cryptococcus castellanii pulcherrima</i> • <i>Rhodotorula pulcherrima</i> • <i>Saccharomyces pulcherrimus</i> • <i>Cryptococcus castellanii</i>
Class	Saccharomycetes	
Order	Saccharomycetales	
Family	Metshcnikowiaceae	
Genus	<i>Metschnikowia</i>	
Species	<i>Metschnikowia pulcherrima</i>	

Yeasts in the genus *Metschnikowia* were first described by Metschnikoff as *Monospora bicuspidate* in 1901 (cited in [6]). The yeast's spore was also observed to be uniquely needle-shaped when the culture was invaded of parasitic species. The genus *Metschnikowia* was then proposed later by Kamienski in 1899 to describe yeasts that possess this needle-shaped spore property. It was put in genus *Candida* as *C. pulcherrima* along with its close relative, *C. reukaufii*, and later placed in the genus *Metschnikowia* by Pitt and Miller (1968) after the suggestion of Wickerham's study in 1964 [7].

M. pulcherrima is known to produce maroon-red pigment under certain conditions, this pigment has been named pulcherrimin after the yeast (shown in Figure 1.2). It was observed by Beijerinck (1908) that the red pulcherrimin produced in *M. pulcherrima* culture can be increased by adding iron salt. He also suggested that pulcherrimin is

produced as a mechanism to cope with excess iron. This was confirmed by later observation that the heavily pulcherrimin-contained cells are mostly non-viable [8].

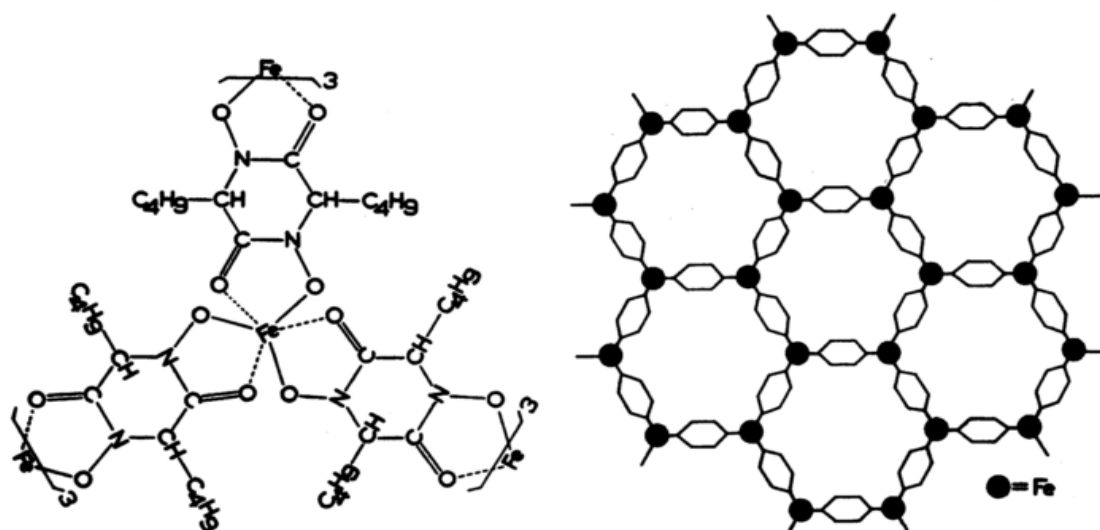


Figure 1.2 Molecular structure of pulcherrimin which is shown to be complex compound of iron and pulcherriminic acid, produced by *M. pulcherrima* (taken from [7]). Pulcherrimin production is one of the key identification mechanisms for *M. pulcherrima*.

M. pulcherrima can reproduce in both asexual and sexual way. While it usually reproduces by budding, it can also do in sexual way under some conditions. *M. pulcherrima* is an ascomycete yeast which means it can produce sexual spores in the vegetative stage. Pitt and Miller (1968) had demonstrated the particular conditions and media types for *M. pulcherrima* to reproduce by creating asci and sexual spores [7].

The distribution of *M. pulcherrima* is worldwide and it can be found on terrestrial areas in almost every continent around the world [9-12]. Though, due to its importance in the wine industry, most reports are centred in the wine producing regions.

Wine fermentation depends greatly on the microbiota that not only aid ethanol production but create a range of flavoursome compounds that give wine its unique smell. Various types of yeasts, including *M. pulcherrima*, are found in the natural grape must and become the starting microorganisms in the wine fermentation. While *Saccharomyces cerevisiae* is the main ethanol producer in the fermentation, it is the other non-*Saccharomyces* yeasts that are the predominant microorganisms in the early stages (Figure 1.3). When the fermentation begins the must has a high sugar content and is highly acidic (pH<3). *M. pulcherrima* is ideally suited to these conditions as well as the apiculate yeasts (*Kloeckera* sp., *Hanseniaspora* sp.) and *Candida* species followed by several species of *Metschnikowia* sp. *Pichia* sp. and could also include *Kluyveromyces* sp., *Schizosaccharomyces* sp., *Torulaspora* sp., *Zygosaccharomyces* sp. and *Brettanomyces* sp.

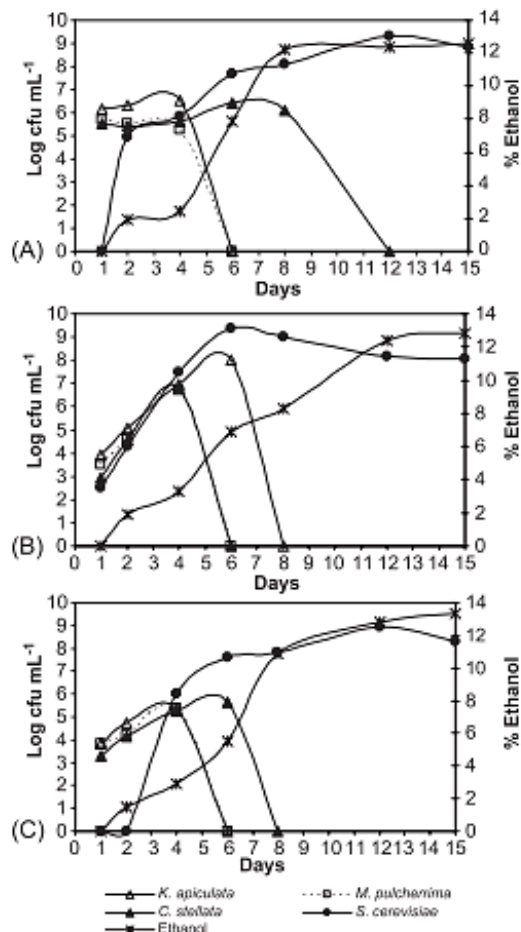


Figure 1.3 Growth of different yeast, including *Kloeckera apiculata*, *Candida stellata*, *Metschnikowia pulcherrima*, *Saccharomyces cerevisiae*, in three wine fermentation samples from Argentina. (taken from reference [13])

As the fermentation progresses, the sugars are consumed and converted to ethanol, albeit it can produce in the low concentration, >3-4 %v/v (Table 1.2). After this point *S. cerevisiae* starts to dominate growth in the fermentation as the pH rises to a more suitable level and the rest of the biota are reduced by the increasing ethanol content. The fermentation then stops when the ethanol concentration is higher than the threshold for the *S. cerevisiae* present.

While non-*Saccharomyces* yeasts are not the main ethanol producers in wine fermentation, they are important for building the aroma characteristics of wine, especially at the early stage of wine fermentation. Wine taste derived primarily from the grapes but secondary tastes and smells come from esters and other aroma compounds produced during fermentation by several microorganisms [13]. The yeasts are known to produce chemical compounds including esters and higher alcohol which give wine aroma. The environmental, agricultural and technological factors, along with grape types, affect the yeast biota in the fermentation and, consequently, the wine quality [14]. *M. pulcherrima* was shown to produce ethyl octanoate and 2-phenylethanol particularly well [15].

Table 1.2 Concentration of major volatile compounds obtained by microvinification of grape must at 18°C for 20 days from isolated selected yeast strains (adapted from reference [16])

Volatile compounds (mg/L)	<i>Candida stellata</i>	<i>Metschnikowia pulcherrima</i>	<i>Saccharomyces cerevisiae</i>
Acetaldehyde	188.0±5.9	73.4±3.81	93.8±0.0
Ethyl acetate	74.8±4.0	62.0±0.30	0
Methanol	33.8±2.5	0	35.5±4.9
2-Butanol	42.4±0.2	0	31.1±0.6
2-Methyl-1-propanol	10.1±2.2	9.3±0.0	7.8±0.7
2-Methyl-1-butanol	36.0±2.5	0	61.6±2.7
3-Methyl-1-butanol	75.7±3.0	0	137.2±7.2
Acetoin	42.3±3.6	11.0±0.2	0
Ethyl octanoate	0	128.7±0.8	0
2,3-Butanediol	16.2±2.9	68.5±1.3	60.9±1.5
2-Phenyl ethanol	35.2±3.9	249.1±6.6	189.9±8.4

M. pulcherrima is also responsible, along with other wine yeasts, in biological control against spoilage wine fermentations. However, while other yeasts may use control mechanisms involving toxins and beta-glucosidase [16, 17], the control mechanism of *M. pulcherrima* seems to be related to iron immobilisation in the media which makes iron-depleted conditions that inhibit other organisms from being able to grow. [3] Moreover, recent study shows interest in *M. pulcherrima* to produce lower alcoholic levels wine production [18].

Due to this ability to control bacterial contamination *M. pulcherrima* has been used as a biological control in a few studies. Its ability to kill off a wide range of bacteria and yeast has been studied since 1991 [19], where it was used against blue and grey mould in apple [20-22], apricot and peach [23]. This property can also be helpful in fermentation in the less sterile conditions in other production interests such as lipid and other metabolic compounds [24].

While some yeasts have been reported as pathogenic, such as *Candida albicans*, and are known to be involved in human infections. Other yeasts including *S.cerevisiae* and *M. pulcherrima* has also been reported to be associated to clinical cases in human, though in much rarer cases [25], and as such can be considered relatively benign.

The ability of *M. pulcherrima* to produce antimicrobials, thereby reducing the chance of invasive species, as well as being able to having pathways to producing valuable compounds, such as 2-phenylethanol, means that it is an extremely promising species for biotechnological applications.

1.2 2-Phenylethanol

2-phenylethanol (2PE) is a higher aromatic alcohol (C_8H_9OH) which has valuable delicate flavour and fragrance with a rose-like odour. It is one of the main bases for perfumes and rose scented food products worldwide. The natural route to 2PE production is usually by extraction from plants and flowers, specifically rose petals. However, the concentration in roses is very small and the process is complicated by difficult extraction protocols that makes it extremely costly. As such 2PE from natural sources can be priced as high as US\$1000/kg. [26] The properties of 2PE is shown in Table 1.3

Table 1.3 Properties of 2-phenylethanol (2PE) [28]

Formula	$C_8H_{10}O$
Molecular weight	122.16 g/mol
Odour perception threshold	In air : 12-24 ng/L In water : 1000 ug/L (orthonasal)
Aspect	Clear colourless liquid
Freezing point	-27°C
Boiling point	219.8°C
Flash point CC	102 °C
Density (20 °C)	1.0202 g/mL
Refractive Index	1.5325
Solubility	2 g/100 mL at 20°C, Soluble in alcohols, esters, aldehydes, benzyl benzoate, mineral oil, chlorinated solvents

The alternative to natural 2PE production is the synthetic chemical 2PE synthesis from the precursors such as benzene and styrene oxide. This is a far more efficient method of production and 2PE produced through these sources costs approximately \$US 5/kg. The current global production of 2PE is estimated at 10,000 tons per year (in 2010), and is mostly from chemical synthesis [26]. However, the removal of by-products in chemical processes which can be presented as green-gassy or metallic-chlorine off-odours in the final products are problematic for production good quality of 2PE [27]. More importantly, uses of chemically synthesised 2PE in cosmetics, beverages and food are restricted [28]. It was reported in 1990 that the 2PE global market was estimated at 7000 tonnes which just 1000 tonnes being used for flavour applications while 6,000 tonnes was used for

fragrance and the rest for downstream chemicals synthesis such as esters. [27] This could suggest that uses in flavour in food and beverage are limited by price, and that a more inexpensive route to natural 2PE production could be highly profitable.

To produce naturally derived 2PE more cheaply, for the production of food and beverages then microorganisms offer a promising route as long as the microorganisms themselves are classed as food grade also. The biochemical pathway for 2PE production in organisms such as plants, bacteria and especially yeasts is well known. 2PE biosynthesis in yeasts concerns 2 sequential pathways which are Shikimate and Ehrlich pathway as shown in Figure 1.4

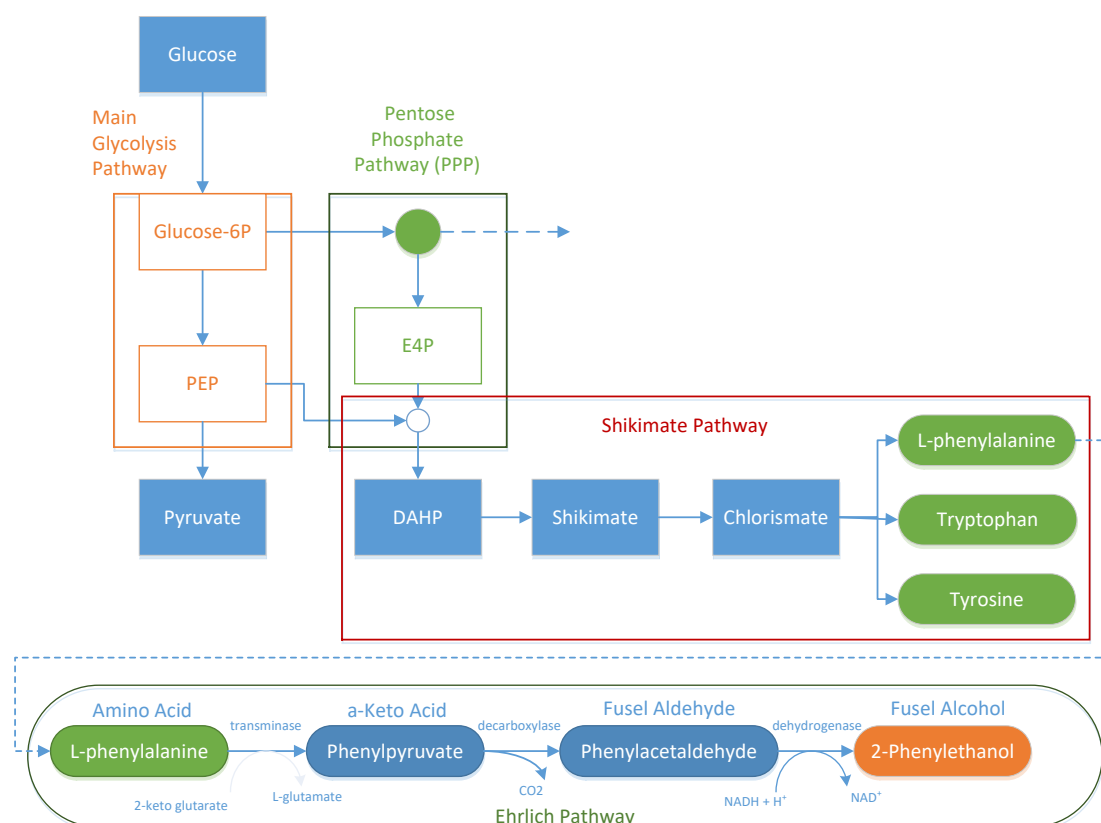


Figure 1.4 Simplified 2-Phenylethanol de novo biosynthesis pathway from glucose via the sequential Shikimate and Ehrlich Pathway. Glucose is converted to aromatic amino acids, including L-phenylalanine. It is consequently converted to 2PE via Ehrlich pathway (adapted from [29, 30]) Abbreviations: PEP (Phosphoenol pyruvate), E4P (Erythrose-4-phosphate), DAHP (3-Deoxy-o-arabiono-heptulosonate-7-phosphate) (Noted it was simplified and could be more complicated with many intermediates and products with a lot of enzymes involved in these pathways, and also back reactions with the main glycolysis as well)

Shikimate pathway is an important pathway to organisms to synthesise aromatic amino acids such as L-phenylalanine, tryptophan and tyrosine from carbohydrate precursors, found in bacteria, fungi and plants [31]. The pathway is not found in animals; therefore these amino acids are essential (phenylalanine and tryptophan needed to be obtained from the diet and tyrosine is converted directly from phenylalanine). In plants, it is the most fundamental biosynthesis pathway to create aromatic compounds needed to build

these aromatic amino acids. Plants create various secondary aromatic metabolites which remain as most of the plants' dry biomass including flavonoids, phytoalexins, indole acetate, alkaloids and lignin. However, the protein synthesis process in bacteria is energy consuming, therefore these amino acids are mostly reserved for building new cells and cell maintenance [29].

When glucose is transported into cells, it is phosphorylated to glucose-6-phosphate (G6P) to preserve glucose in the cells and prevent the back-transport. Consequently, it is metabolised through glycolysis and yields pyruvate which can be metabolised further. G6P is also partially metabolised through the Pentose Phosphate Pathway (PPP), normally used for creating important proteins such as nucleotides, coenzymes, DNA and RNA via intermediates such as ribulose-5-phosphate.

To produce aromatic amino acids through the Shikimate pathway, erythrose-4-P (E4P), one of the intermediates produced in the PPP, and phosphoenolpyruvate (PEP), which is the intermediate in the main glycolysis pathway, combine to yield DAHP. This compound is then further converted through several intermediates and with many enzymes involved through shikimate and chorismate to yield aromatic amino acids including phenylalanine, tryptophan and tyrosine.

In yeasts, amino acids are mostly preserved for cell maintenance and building new cells as well because of its non-autotrophic nature. However, yeasts seem to possess the ability to convert those amino acids into aldehydes, higher alcohols and acids. This ability had been observed and reported a century ago by Ehrlich [32]. Amino acids that can be converted by yeasts through Ehrlich pathway include the branched-chain amino acids (valine, leucine, isoleucine), sulfur-containing methionine and aromatic amino acids (phenylalanine, tyrosine, tryptophan) which yield α -keto acids, fusel aldehydes and fusel alcohols as shown in Table 1.4 [33].

When L-phenylalanine is the precursor, it can be transaminated to phenylpyruvate, then decarboxylated to 2-phenylaldehyde and reduced to 2-phenylethanol as the end product. In some organisms, fusel alcohols can be further converted to fusel acids. In such case, 2PE can be converted to 2-phenylacetate.

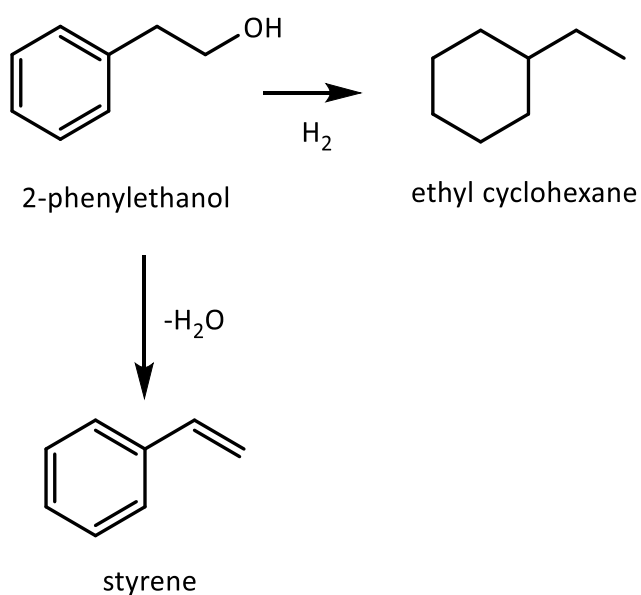
Table 1.4 The α -Keto acids, fusel aldehydes and alcohols converted from each amino acids through Ehrlich Pathway [33]

<i>Amino Acid</i>	<i>α-Keto acid</i>	<i>Fusel aldehyde</i>	<i>Fusel Alcohol</i>
<i>Leucine</i>	<i>α-Ketoisocaproate</i>	<i>Isoamylaldehyde</i>	<i>Isoamyl alcohol</i>
<i>Valine</i>	<i>α-Ketoisovalerate</i>	<i>Isobutanal</i>	<i>Isobutanol</i>
<i>Isoleucine</i>	<i>α-Ketoethylvalerate</i>	<i>Methylvaleraldehyde</i>	<i>2-Methylbutanol</i>
<i>Phenylalanine</i>	<i>Phenylpyruvate</i>	<i>2-Phenylacetaldehyde</i>	<i>2-Phenylethanol</i>
<i>Tyrosine</i>	<i>p-Hydroxyphenylpyruvate</i>	<i>p-Hydroxy phenylacetaldehyde</i>	<i>Tyrosol</i>
<i>Tryptophan</i>	<i>3-Indole pyruvate</i>	<i>3-Indole acetaldehyde</i>	<i>Tryptophol</i>

2-PE and other higher alcohols have been observed in yeast fermented food and beverages, particularly wine [34, 35]. The difference of mixture of these aroma compounds would affect greatly on the delicate tastes, flavours and fragrances in wine which can mark the good quality in wine. As the quality of wine is highly priced and sought after, much research has been devoted to understand how yeast produces these flavours and fragrances. Currently it is well studied and sufficiently documented to understand the biochemical pathways in yeast fermentation in wine and other products and their importance [36].

1.3 Alternative uses for 2PE

While 2PE is currently only used as a flavour and fragrance there are potentially other markets that could be exploited with a more economic process. For example, bioplastics and biofuels have been heavily researched in the last few decades. 2PE is aromatic and as such could be used as a precursor in the production of aromatics biopolymers (scheme 1.1). For example, a simple dehydration reaction would give styrene, the monomer of polystyrene, produced on the million tonne scale presently from fossil resources. In addition, aromatic based biofuels, necessary in the aviation industry or the higher value bio-additive market could be accessible to a lower cost 2PE product. For example, the octane booster ethyl cyclohexane could be produced through the hydrogenation of 2PE. While these compounds are presently far too inexpensive to be realistic targets for a bio-derived 2PE product, it demonstrates that with increasing bulk there would be more markets available if the 1,000 – 10,000 tonne a year fragrance market becomes saturated.



Scheme 1.1 – Possible products from 2-phenylethanol

1.4 Advances in *in situ* 2PE removal fermentation

2PE can be produced in yeast from glucose through pentose phosphate pathway and shikimate pathway to yield L-phenylalanine which subsequently be converted to 2PE through Ehrlich pathway. However, carbon flux through shikimate pathway and pentose phosphate pathway is complex and usually governed by stage of growth and culture condition such as nitrogen limitation [37]. Therefore, 2PE is present in quite low titres in typical yeast fermentation products such as wine and beer fermentation. The low 2PE concentration by the traditional yeast fermentation may not be economical and repel 2PE production from glucose.

One popular approach in the 2PE production by yeast is the bioconversion from external L-phenylalanine as most yeasts are capable of convert L-phenylalanine to 2PE via Ehrlich pathway [38]. The critical enzymes governing in 2PE conversion in Ehrlich pathway, such as 2-Keto-acid decarboxylase (KDCs) which decarboxylate phenylpyruvate to phenylaldehyde are commonly found in yeast, fungi and plants but less so in bacteria, and the alcohol dehydrogenase (ADHs) which convert the aldehyde to 2-phenylethanol are found in most organisms. [39] Therefore, bioconversion from L-phenylalanine can be studied as the first step of 2PE production at a larger scale.

Table 1.5 Maximum 2-phenylethanol biosynthesis by different yeast strains in molasses medium with L-phenylalanine 7 g/L at 35 °C after 41 hr [38].

Strain	2-PE (g/L)	% of theoretical maximum
<i>Kluyveromyces marxianus</i> CBS 600	0.89	17.2%
<i>Kluyveromyces marxianus</i> CBS 397	0.84	16.2%
<i>Zygosaccharomyces rouxii</i> CBS 5717	0.8	15.4%
<i>Kluyveromyces marxianus</i> DSMZ 4906	0.76	14.7%
<i>Kluyveromyces marxianus</i> CBS 6432	0.73	14.1%
<i>Kluyveromyces marxianus</i> CBS 5670	0.5	9.7%
<i>Saccharomyces cerevisiae</i> DSMZ 70487	0.34	6.6%
<i>Clavispora lusitaniae</i> DSMZ 70102	0.33	6.4%
<i>Kluyveromyces lactis</i> DSMZ 70793	0.29	5.6%
<i>Pichia anomala</i> DSMZ 70130	0.19	3.7%
<i>Pichia membranaefaciens</i> CBS 8427	0.13	2.5%
<i>Pichia membranaefaciens</i> CBS 637	–	
<i>Saccharomyces cerevisiae</i> NCYC 739	–	
<i>Schizosaccharomyces pombe</i> DSMZ 70572	-	

Several non-saccharomyces yeasts, usually found in the wine fermentation were studied for their 2PE biosynthesis from L-phenylalanine by Etschmann et al. (2003), the results are summarised in Table 1.5 [38]. This work demonstrated that many selected yeasts can convert L-phenylalanine to 2PE, presumably through Ehrlich pathway. *Kluyveromyces marxianus* was found to be the most prominent producing the highest 2PE under the conditions examined (0.89 g/L). It also shows that not all strains in some

yeasts such as *Pichia membranaefaciens* and *Saccharomyces cerevisiae*, can produce 2PE from L-phenylalanine.

Theoretically, 1g of L-phenylalanine can yield 0.74 g/L of 2PE, by molar basis with 100% conversion, therefore the best results presented above achieved approximately 17% of this total. Many recent studies have attempted to use yeasts that can convert more L-phenylalanine by screening strains and inducing mutation. Wang et al. (2008) screened 45 industrial yeast strains for biosynthesis 2PE from surplus L-phenylalanine supply and found *S.cerevisiae* up to 1.7 g/L and up to 4-5 g/L in a fed batch study [40]. Selected UV radiated *S. cerevisiae* mutants were also found to increase slightly 2PE production by approximately 10% [41, 42].

Cytotoxicity of 2PE to yeasts is the important factor that can limit the production of 2PE from biosynthesis. Etschmann (2003) suggested that 2PE completely inhibits *S. cerevisiae* and *K. marxianus* at 4.0 and 2.0 g/L, respectively. This toxicity has been confirmed by other authors as well [43, 44]. In order to produce 2PE in a higher volume, therefore, it is necessary to keep 2PE concentration far below this inhibitory level.

Many in-situ product removal (ISPR) techniques have been studied in 2PE biosynthesis, to increase these titres. These include liquid-liquid extraction, adsorption, reverse osmosis membranes, solvent immobilisation and organophilic evaporation. The most common ISPR technique for 2PE fermentation is probably the liquid-liquid separation. For *in situ* liquid-liquid 2PE removal, an insoluble non-toxic solvent can be added in the fermentation broth without harming the microorganism. Several organic solvents have been used such as Isopropyl myristate, miglyol, oleyl alcohol [38] and oleic acid [40, 45, 46]. Stark et al. (2002) reported 2PE biosynthesis from excessive L-phenylalanine as precursor in *S.cerevisiae* with oleyl alcohol as ISPR increased overall 2PE production to 12 g/L [46].

Several adsorbents have also been studied as ISPR in 2PE biosynthesis. Various non-polar macroporous resins have been tested for the 2PE adsorption capacity and compatibility with the bioconversion. Gao and Daugulis (2009) reported HytreI® resin is the best in 2PE adsorption, among other organic polymer and solvents including Nylon, HDPE, 1-dodecene, 1-dodecane, PPG1000 and polyurethane [46]

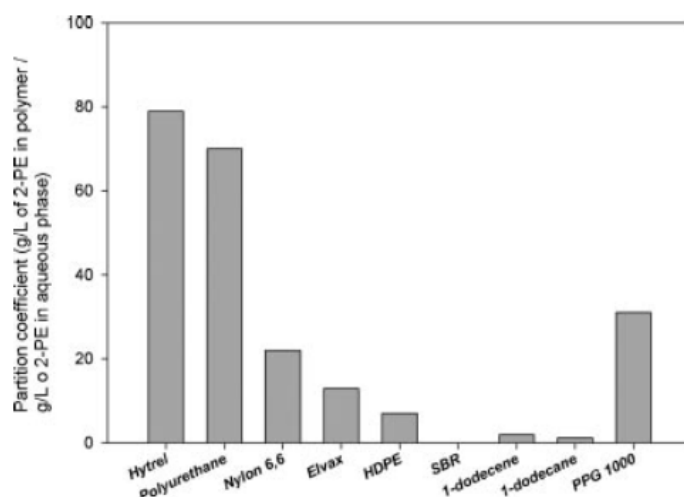


Figure 1.5 Partition coefficient of 2PE in organic solvents and polymers sorbents, calculated from slope plotted between 2PE concentration in aqueous phase and solvent phase with the initial 2PE concentration 2, 3, 4, 5 g/L (taken from reference [47])

Hytrel® adsorption resin was then used as ISPR adsorbent (500g) in 2PE biosynthesis by *K.marxianus* in a 3L bioreactor [47], this produced an impressive overall yield of 13.7 g 2PE/ L. Further to this study, Hua et al. (2010) studied 2PE adsorption in several adsorption non-polar resins, such as CAD40, CD180, DM11, HZ801, HZ818, HZ841, Amberlite XAD7HP and Amberlite XAD1180, and found that 2PE adsorption capacity for those resins are limited and fell between 0.01-0.14 g/g where DM11, CAD140, XAD1180 and HZ818 are better with adsorption capacity higher than 0.075 g/g with HZ818 is the highest at 0.14 g/g.

Other separation technique such as membrane technology have also been used for the 2PE biosynthesis. Adler et al. (2011) reported the biosynthesis from l-phenylalanine using a cross-flow hollow fibre module included in the process [48]. From this work, 4 g of 2PE/L and a significant quantity of phenylacetate was produced from the *K. marxianus* fermentation. Following this study, Mihal et al. (2013) experimented with using a hybrid air-lift bioreactor with immersed membrane module. This yielded extremely high 2PE conversions and a volumetric production of 18.6 g/L [49-51].

1.5 Other compounds from *M. pulcherrima* fermentation

Microorganisms generally produce a range of various metabolic compounds during a fermentation these are either intermediate compounds, used by the organism for some function later on or are by-products that can be collected. While this can move flux away from the target products of a fermentation, if the secondary metabolites also have value this can be used to reduce the cost of both products. *M. pulcherrima* has been reported to produce two other main co-products alongside 2-phenylethanol which are arabitol and lipids.

1.5.1 Arabitol

Arabitol (sometimes called arabinitol) is a C₆ sugar alcohol that is not normally found in the vast majority of yeast fermentations. It is an enantiomer of xylitol which is popularly used as a substitute sweetener in several commercial products such as dental gum as it does not lead to dental cavities.

Xylitol is the far more common C₆ sugar produced, and biochemically is produced mainly from yeasts in the *Candida* glade. The first industrial xylitol production was in Finland, started in 1975. It was mostly done by chemical processes in the reduction of xylose isolated from birch trees. However, the new approach for more sustainable practice led to the development of the biochemical route which could produce xylitol, and potentially arabitol from glucose.

While still lagging far behind xylitol production, arabitol production has recently received attention as another attractive bioproduct which can be used as a sweetener. It has a much lower calorific value than xylitol so is potentially more attractive as an artificial sweetener. It has also been investigated as a precursor for downstream production of a range of compounds. For example, xylitol and arabitol are included in 12 building block chemicals for the next generation bioproducts conducted by the US Department of Energy [38]. The report summarised 12 chemicals, including xylitol and arabitol that could be used as a building block for sustainable products in the future which can be produced from biomass. Some work has gone on producing chiral polymers from xylitol and as such arabitol offers an interesting co-monomer to help develop further interesting polymer properties. [52] Arabitol can also be converted into a range of useful compounds such as arabonic acid, propylene glycol and ethylene glycol [53].

The production of L-arabitol have been studied since 1970s in several yeasts such as *Candida tropicalis* and *Pachysolen tannophilus* can metabolise L-arabinose to L-arabitol. [39] On the other hand, D-arabitol can be produced from glucose which is more commonly found. Most yeast that can produce D-arabitol are osmophilic yeasts in genera

1.5.2 Lipid

Biofuel has been one of the major focus in chemical engineering research in the last few decades. Biofuels such as ethanol have been widely studied and been demonstrated on an industrial scale to be a commercially viable substitute for gasoline. However, other biofuels such as biodiesel has not achieved the similar success due to limitation in raw materials such as the vegetable oils, rapeseed oil, sunflower oil, palm oil and soybean oil [60].

Biodiesel is the fatty acid methyl esters produced from the transesterification of vegetable oil and methanol. Its properties are close enough to diesel fuel that it can be used in compression ignition engines, though currently only in blends of up to 7% with commercial diesel fuel. However, the use of fuel derived from food raised concerns over increasing food price due to competition for biodiesels raw material and the ethical question of unfair distribution of food. In addition, the increase of land used for soybean and palm oil plantation for the increasing demand of vegetable oil for biodiesel production can cause deforestation, particularly in the Asian and Amazon tropical rainforests. Therefore, the focus of biodiesel production has been shifted to other source for lipid that has less impact on the environment such as oil from microalgae or yeast [61-64].

Oleaginous yeasts are a group of yeasts that can accumulate intracellular lipid typically over 20% of biomass weight. They were originally thought only handful 20-30 species of yeast are oleaginous [65, 66] but recently many have been reported up to 70 species [67]. The oleaginous yeast that have been reported are mostly ascomycetous yeast in genera *Lipomyces* [68] and *Yarrowia* [69-71] and basidiomycetous oleaginous yeasts in general *Rhodotorula* and *Rhodospiridium* [72]. Recently the lipid study in yeasts are increasing and the knowledge in lipid from yeast and fungi are more sophisticated. Now the knowledge of lipid products is expanded and wider range of lipid produced by oleaginous yeast is known. Not only for vegetable oil substitution, some valuable nutritional oil such as docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), gamma linolenic acid (GLA) and arachidonic acid (ARA) are known to be achieved by different types of microorganisms. Different quality of oils from different microorganisms have been more documented [67].

Lipid production in yeast generally occurs where the nitrogen source is limited and carbon is available. It leads yeast to change its metabolism from nitrogen-based protein synthesis, for example, cell building in the growth phase to the organic compounds synthesis without nitrogen such as lipid. Yeast mostly produce similar oils and fatty acids found in plants such as oleic acid (18:1), palmitic acid (16:1), linoleic acid (18:2) and stearic acid (18:0) [73]. Other nutritional polyunsaturated fatty acids such as which can be produced in the useful amount by molds and algae [74].

Intracellular lipid in oleaginous yeasts are mostly in the form of droplets inside cells, mostly triacylglycerols (TAG). However, other cellular lipids that are functional in cells also generated such as phospholipids, sterols and sphingolipids. Usually phospholipids are the major lipid class in lipid production as it is the major component of cell membrane. Lipid synthesis in yeast cell generally starts with synthesis of free fatty acids (FFA). FFAs can be synthesised from various substrate such as *de novo* synthesis from carbohydrate, external uptake from extracellular sources or lipid degradation. Modern genetic studies have revealed certain enzymes govern with the FFA synthesis and degradation, this is depicted in Figure 1.7 and Figure 1.8

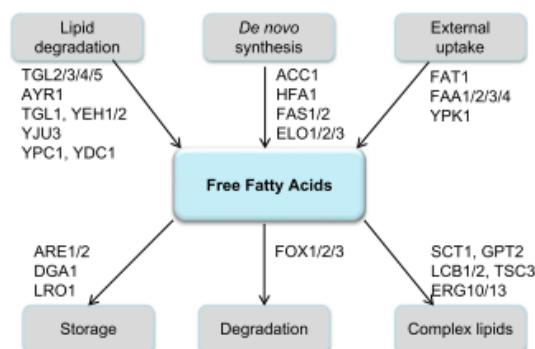


Figure 1.7 Fatty acids metabolisms in *Saccharomyces cerevisiae* (taken from [77])

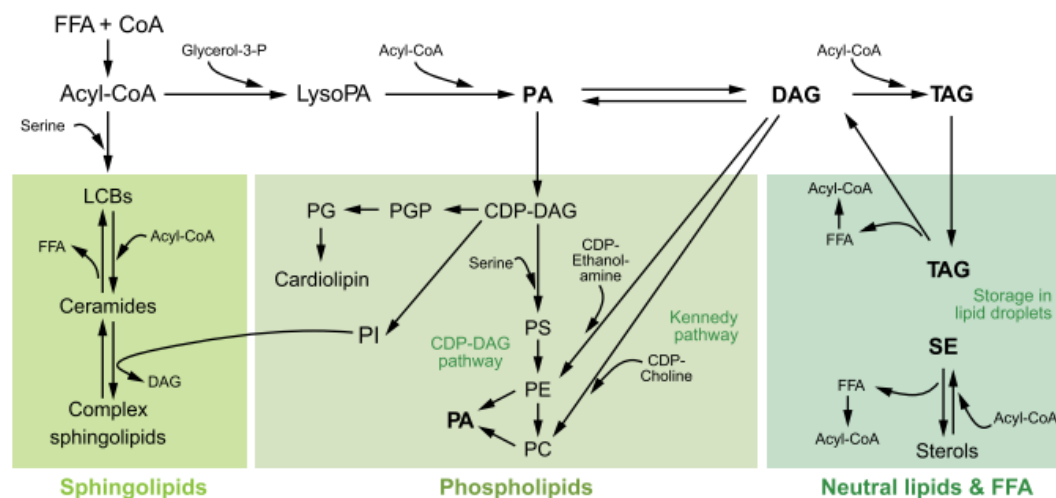


Figure 1.8 Simplified overview of selected aspect of yeast lipid metabolism including lipid synthesis from fatty acids for neutral lipids (storage in intracellular oil droplet), phospholipids and sphingolipids. The main metabolite phosphatidic acid (PA) is generated from Acyl-CoA, which are from free fatty acids (FFA) and Coenzyme-A (CoA), and Glycerol-3P. PA is the main precursor to synthesise diacylglycerols (DGA), triacylglycerols (TGA) and phospholipids. SE is steryl ester stored in intracellular oil droplet as well. (taken from [75])

The lipids that are accumulated in oleaginous yeasts are neutral lipids which are mostly triacylglycerols (TAG) stored in oil droplet in the oleaginous cells. They are synthesised with free fatty acids (FFA) as a precursor. FFAs are converted together with coenzyme-A (CoA) yielded Acyl-CoA. It can be converted to sphingolipids and, together

with glycerol-3P, generates phosphatidic acid (PA) which is the main metabolite in diacylglycerols (DAG) and TAG synthesis. PA can then be synthesised phospholipids and neutral lipid through DAG and TAG synthesis.

Metschnikowia pulcherrima is an ascomycetous yeast which is known for oil accumulating ability for more than a century. It was one of the first reports on yeast that accumulate lipid since Lindner observed that *Torula pulcherrima* (synonym to current *M. pulcherrima*) can accumulate intracellular fat [76]. However, the lipid production study in genus *Metschnikowia* is rather limited and other genera of ascomycetous yeast such as *Yarrowia sp.* have received more attention [77]. However, it has been recently demonstrated that oil produced from *M. pulcherrima* is similar to palm oil, therefore, it could be used for palm oil substitution and reduce the pressure on deforestation by palm oil cultivation [5]. In addition, its advantages include it can be cultured for lipid production in the non-sterile condition [78].

While *M. pulcherrima* has great potential as a novel yeast for biotechnology the development of 2PE production from this yeast has not been investigated nearly as much as the lipid or arabitol production, where both processes have been patented [53, 79].

1.6 Aims and objectives

The aim of this work was to assess *M. pulcherrima* to produce 2PE. It was also hypothesised that the bioconversion synthesis and *in situ* production modes, as well as continual removal will increase the 2PE production titre in the process. The proven robustness of *M. pulcherrima* would be an excellent platform organism for the 2PE production, and in this way, achieve a more suitable, economically viable process.

In the second chapter, the de-novo production was assessed with a synthetic grape juice media on the lab scale, the conditions were then optimised to produce 2PE. In the third chapter this *de novo* production was scaled up to a 2L stirred bioreactor system and the 2PE production optimised. In the fourth chapter the ability of *M. pulcherrima* to convert phenylalanine to 2PE was assessed. Finally, in Chapter 5, to produce the highest titres possible, a continual extraction and continuous mode running in 2L controlled bioreactors was then investigated using the in-situ conversion of phenylalanine.

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Chapter 2

De novo 2-Phenylethanol Production
and Other Co-Products from
Metschnikowia pulcherrima
in Batch Culture

2.1 Introduction

Fragrance is essential in food, perfume and personal care products. Traditionally these valuable compounds can be retrieved by extraction from flowers and natural sources which usually require large number of natural flowers and spices where they occur in low concentrations. The yield from natural extraction therefore are very low which makes the naturally sourced fragrances highly expensive. One of the highest produced single fragrances is 2-phenylethanol which gives rose-like aroma and is one of the popular and sought after in the industry. As such, naturally produced 2-phenylethanol extract costs upwards of 1000\$ kg⁻¹ [26].

2-phenylethanol (2PE) can be produced biochemically in microorganisms via the Ehrlich metabolic pathway which has L-phenylalanine as a precursor. It also includes the biosynthesis of alternative higher alcohols with amino acids as precursors as well.

Another pathway governs the *de novo* synthesis of L-phenylalanine when L-phenylalanine is scarce, some microorganisms can synthesis L-phenylalanine through the Shikimate pathway that produce aromatic amino acids from glucose via pyruvatealdehyde. It links the metabolism between carbohydrate and amino acid production in several organisms, particularly plants and microorganisms.[80] Essential aromatic amino acids, such as L-phenylalanine, L-tyrosine and L-tryptophan are created via this pathway. This is mostly for the synthesis of polypeptides and other activities such as creating new cells. These amino acids can be transformed to higher alcohols such as 2-phenylalanine, tyrosol and tryptophol via the Ehrlich pathway. [81, 82]

Several yeast, mainly in the genera *Kluyveromyces*, *Zygosaccharomyces*, *Saccharomyces*, *Hansenula*, *Pichia*, *Candida* and *Clavispora* are reported to be able to produced 2PE [83]. However, 2PE production from these yeasts are low, usually in the 10-100mg/L range via *de novo* synthesis (synthesis from glucose without additional L-phenylalanine as a precursor). Therefore, it had been long considered that the *de novo* synthesis of 2PE is not economically viable and a larger focus has rested on the biosynthesis from L-phenylalanine to 2PE instead [30].

Metschnikowia pulcherrima is naturally found on flowers and fruits skin and known as non-Saccharomyces yeast that produce the wine aroma at the early stage of wine production while the wine must be acidic. While 2-PE can be produced by other yeasts via biosynthesis with the L-phenylalanine amino acid as a precursor, *M. pulcherrima* can produce compound the via *de novo* synthesis from glucose which is vastly less costly than from L-phenylalanine. With technology of cellulose depolymerisation which yields glucose and other sugars, producing 2-PE from glucose is prominently more attractive than from L-phenylalanine.

Study of 2PE production are mostly started in wine fermentation study because the condition of wine fermenting allows the yeasts to generate aromas in wine. The wine must for starting wine fermentation has high sugar content, high osmotic pressure, limited nutrients and low pH. So this study will start with the synthetic media that imitate the wine must in wine fermentation.

2.2 Media selection

It is reported that *M. pulcherrima* produces a large range of alcohols, esters and aromatic compounds in wine production [84]. To assess which promising products were produced from *M. pulcherrima*, the standard strain obtained from the National Yeast Collection Centre was cultured on a synthetic grape juice media (SGJ media). This media was formulated to replicate the grape juice from wine fermentation. SGJ media was composed of high concentrations of monosaccharide sugars (glucose 70 g/L and fructose 30 g/L) and the organic acids tartaric acid (7 g/L) and malic acid (10 g/L). Limited nitrogen and micronutrients were added to imitate the natural wine must, the C/N ratio was set at 550:1. To study the effect of the limited nitrogen to the *M. pulcherrima* fermentation, a modified synthetic grape juice media (MSGJ media) which had an increased nitrogen content (C/N ratio of 100:1) was also used.

M. pulcherrima grew well on both SGJ and MSGJ with an extremely short lag phase. In SGJ media, the yeasts growth rate and the glucose utilisation rate is fast in the first 2 days, the optical density increased from 0 to 11 units though the rate declined after day 3 (Figure 2.1 a). This is presumably due to the lack of available nitrogen in SGJ media. When the yeast starts growing, glucose and nitrogen are used rapidly for cell reproduction, after day 3 the nitrogen source is depleted, the cell reproduction and sugar utilisation rate is slower. In comparison when *M. pulcherrima* was cultured in MSGJ, an identical media except with a higher C/N ratio of 100:1, the glucose is utilised faster in the log phase (Figure 2.1 b). Statistical t-test analysis of the yeast growth showed that *M. pulcherrima* grew significantly better in MSGJ than in SGJ. By day 6, the glucose was depleted and the yeast had entered the stationary phase. The maximum biomass of *M. pulcherrima* in SGJ media was approximately 5 g/L less than in MSGJ media. This demonstrates that the nitrogen source is important for the growth and the maximum cell concentration of *M. pulcherrima*. The C/N ratio of 100:1 seemed to be sufficient for the required growth of *M. pulcherrima*.

However, in MSGJ media which the yeast grows quickly, the growth rate and the sugar utilisation rate between day 2 to day 4 fluctuated slightly which may be due to the presence of inhibitors, possibly ethanol, which can be produced by *M. pulcherrima* under anaerobic conditions. As the yeast grows very quickly in the first day, the yeast metabolises using an aerobic pathway. When available oxygen is used up and the anaerobic condition

occurs, *M. pulcherrima* changes to an anaerobic pathway and potentially produces ethanol as a side product, this is known even at low concentrations to inhibit the growth of *M. pulcherrima* [85].

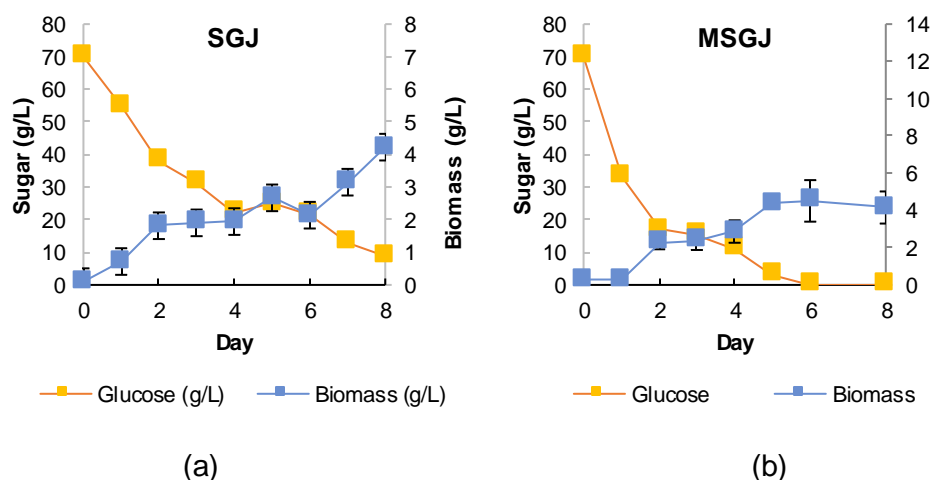


Figure 2.1 Utilisation of substrate in the fermentation in SGJ media (a) and modified SGJ (C/N 100:1) (b) and growth of *Metschnikowia pulcherrima* and sugar utilisation in fermentation in SGJ media (a) and modified SGJ (C/N 100:1) (b) (number of culture replica, n , =3, error bars represent one s.d.)

The utilisation of substrate in the fermentation was monitored by HPLC. From the HPLC analysis, all substrates except tartaric acid were found to be metabolised by *M. pulcherrima*. Tartaric acid acts as pH buffer to keep the constant pH and used as the internal standard in the HPLC analysis because it cannot be metabolised by the yeast. In SGJ media, the substrates utilisation rates are gradual, presumably due to the limited nitrogen source (Figure 2.2). *M. pulcherrima* can use both glucose and fructose, though the glucose utilisation rate is twice higher than the fructose utilisation rate ($0.286 \text{ g/g} \cdot \text{d}^{-1}$ for glucose, $0.105 \text{ g/g} \cdot \text{d}^{-1}$ for fructose). However, malic acid is only metabolised when the concentration of glucose and fructose are much reduced. In contrast, in MSGJ media, which has more available nitrogen, the substrate is metabolised much more quickly and is consumed within the first 6 days (Figure 2.2 b). Similar to SGJ medium, the rate of glucose utilisation in MSGJ is twice higher than fructose utilisation ($0.444 \text{ g/g} \cdot \text{d}^{-1}$ for glucose and $0.226 \text{ g/g} \cdot \text{d}^{-1}$ for fructose). The rate of total sugar utilisation in MSGJ medium in the growth phase is $0.446 \text{ g/g} \cdot \text{d}^{-1}$, compared to $0.286 \text{ g/g} \cdot \text{d}^{-1}$ in SGJ medium.

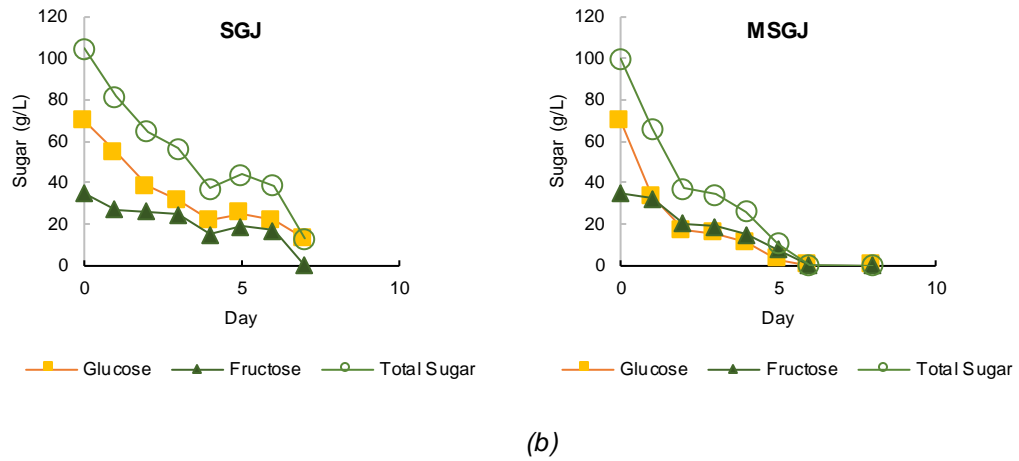


Figure 2.2 Utilisation of substrates in the fermentation in SGJ media (a) and modified SGJ (C/N 100:1). Total sugar is the amount of glucose and fructose combined.

The necessity of the nitrogen, phosphorus and organic acids in the culture of *M. pulcherrima* in SGJ media was examined using four types of SGJ media where the nitrogen source, phosphorus source, tartaric acid and malic acid were removed from the media, with the normal SGJ media as control (Figure 2.3).

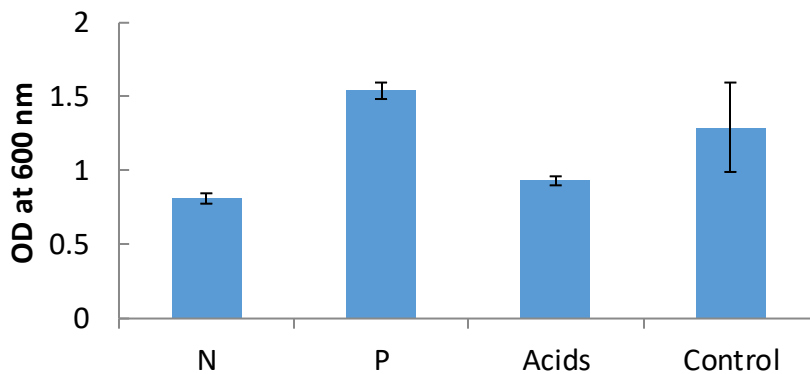


Figure 2.3 Effects of the depletion of nitrogen, phosphorus and acids on the growth of *M. pulcherrima* in SGJ media. The amount of compounds were reduced to 1/10 of regular formula in the depletion study ($n=3$, error bars represent one s.d.)

The cell density in SGJ media without nitrogen source demonstrated the lowest growth. This confirmed that an appropriate level of nitrogen is vital for the growth of *M. pulcherrima*. Interestingly the depletion of phosphorus does not affect the growth of *M. pulcherrima*, and there is presumably more than enough in the inoculum for growth. The growth of *M. pulcherrima* is also effected by the removal of tartaric acid and malic acid, even though the yeast cannot metabolise tartaric acid. *M. pulcherrima* can metabolise malic acid, though the media was rich in glucose and fructose which is more readily metabolised. However, tartaric acid and malic acid can act as pH buffer in the media and presumably when there are no acids, the change of pH adversely affects the growth of *M. pulcherrima*.

2.3 Assessment of fermentation products formed in the fermentation

To assess the suitable fermentation products from the yeast the supernatant was sampled every 24 hours for HPLC analysis. 2-PE was observed in the cultures, coming at retention time 3.14 min in HPLC analysis with C18 column and UV detector at 216 nm, this was confirmed by GC-MS analysis.

During the growth period, the yeast need the aromatic amino acids group such as L-phenylalanine or L-tryptophan to create biomass, it can produce them *de novo* from glucose via shikimate pathway which yields phenylpyruvate as an intermediate compound which can be converted to L-phenylalanine but it can also be a precursor to produce 2-phenylethanol as well. So while the yeast produces L-phenylalanine to synthesise protein chains for new cell and cell growth, it also produces 2PE as well. However, when the yeast reaches the stationary phase, it is less necessary to produce protein, which may act as a trigger to the cell to switch off the shikimate pathway, reducing the production of phenylpyruvate and consequently reducing the production of 2PE. The fact that 2PE is produced mainly during the growth period can be useful for reactor design reducing the cost of unnecessary aeration and fermentation time.

From the HPLC analysis, a further range of unknown metabolites were observed alongside 2-PE (Figure 2.4 and Figure 2.5). The unknown metabolites are named here with a letter followed by their HPLC retention times as a subscript e.g. the unknown compound C_{10.6} is the metabolite with HPLC retention time of 10.6 minutes. Because tartaric acid is an acid in the media with known quantity and not metabolised by *M. pulcherrima*, it was used as a reliable internal standard in the analysis and % area of tartaric acid is used to refer to the quantities of unknown products.

While *M. pulcherrima* does not grow as well in SGJ media, the yeast produces a higher proportion of fermentation products A_{7.1}, B_{7.8}, C_{10.6}, E_{12.5}, F_{14.4}, G_{15.8}, I_{17.1}, J_{18.2} and ethanol.

Ethanol production in non-Saccharomyces wine yeasts is common, and most yeasts produce ethanol under anaerobic conditions. Some wine yeasts, including *Saccharomyces cerevisiae*, can produce ethanol even under aerobic conditions, this is known as the 'Crabtree effect'. While *M. pulcherrima* is known to produce ethanol, though not in high titres, the yeast also tends to consume ethanol as a substrate as well. In fact, *M. pulcherrima* produces lower ethanol than other yeasts and is sometimes selected to reduce ethanol in specialist wine fermentations [86, 87].

The most produced metabolite was the unknown compound C_{10.6}. This was produced from the start of the fermentation and was found to constantly increase as the

fermentation proceeded. Interestingly, the production of C_{10.6} in MSGJ media, is noticeably lower and is only about 40% of the C_{10.6} produced in SGJ media.

One possible explanation is that the yeast in MSGJ media has enough nutrients for cell reproduction and most of the carbon source is changed into biomass. But in SGJ media, the cell reproduction and biomass generation is limited; therefore, the available carbon source is converted into fermentation products instead of creating biomass.

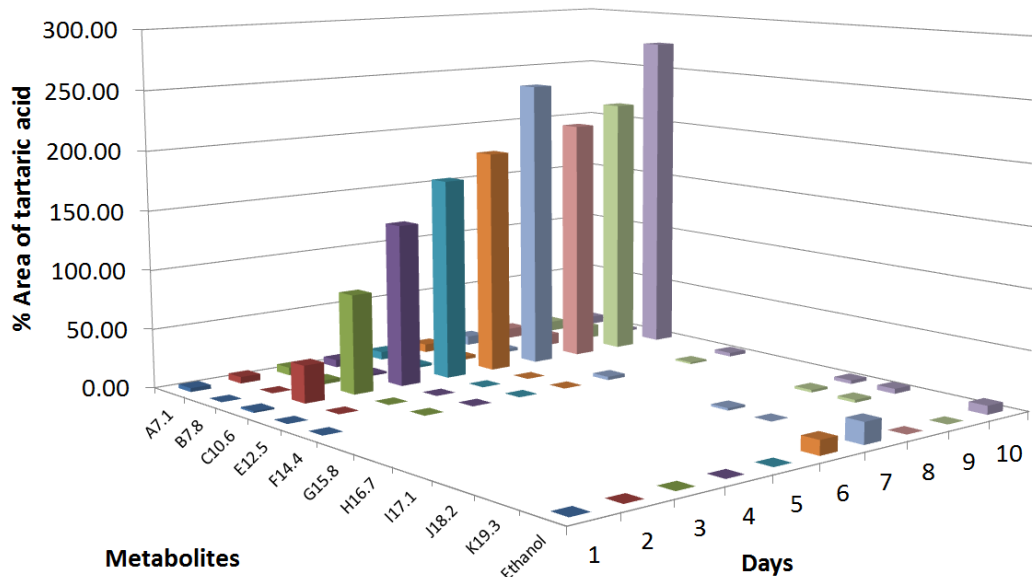


Figure 2.4 Fermentation products from *M. pulcherrima* in SGJ media from HPLC analysis

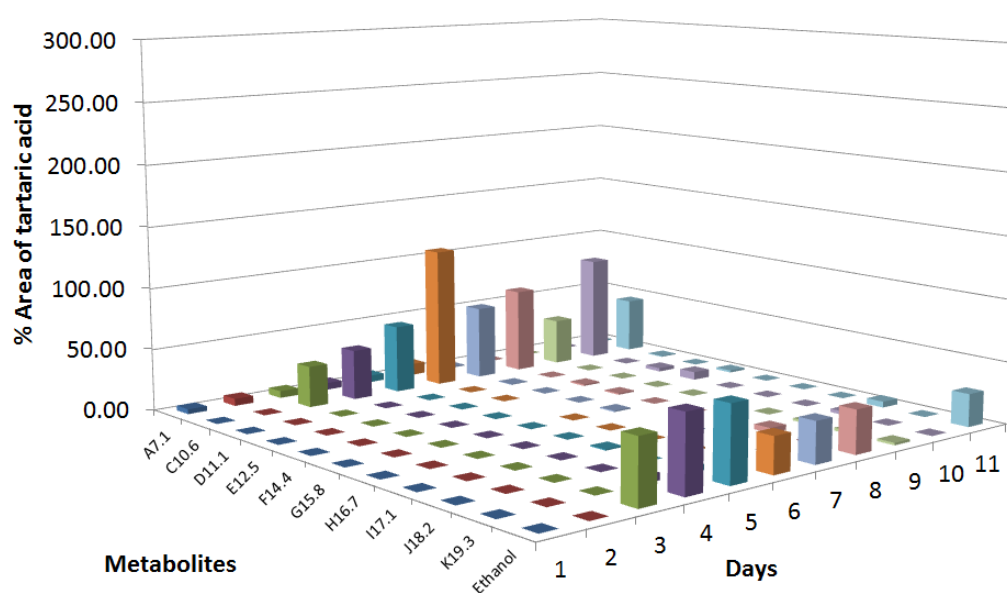


Figure 2.5 Fermentation products from *M. pulcherrima* in MSGJ media from HPLC analysis

To determine the identity of the peak, a sample of the supernatant was fractionated from the HPLC eluent at the retention time of the peak. Mass spectroscopy analysis was done on the pure C_{10.6} compound. The analysis suggests that the compound had the molecular weight of 152.14 and the possible molecular formula of C₆H₁₂O₅. The potential chemicals for this are the C₅ sugars: ribitol, arabitol and xylitol. To identify the product, these three sugars were analysed by HPLC (1 g/L, with tartaric acid 1 g/L as an internal standard) and compared to the supernatant of *M. pulcherrima* fermentation in MSGJ media, pH 4 at 20 °C at the end of the fermentation (Figure 2.6). The chromatogram shows no sugars are left, the peak of tartaric acid (RT 8.1 min), malic acid (RT 9.0 min) and C_{10.6} (RT 10.6 min). The chromatograms of ribitol, arabitol and xylitol are in black, blue and green, respectively. The retention time of the arabitol aligns exactly with C_{10.6} confirming the identity of the compound.

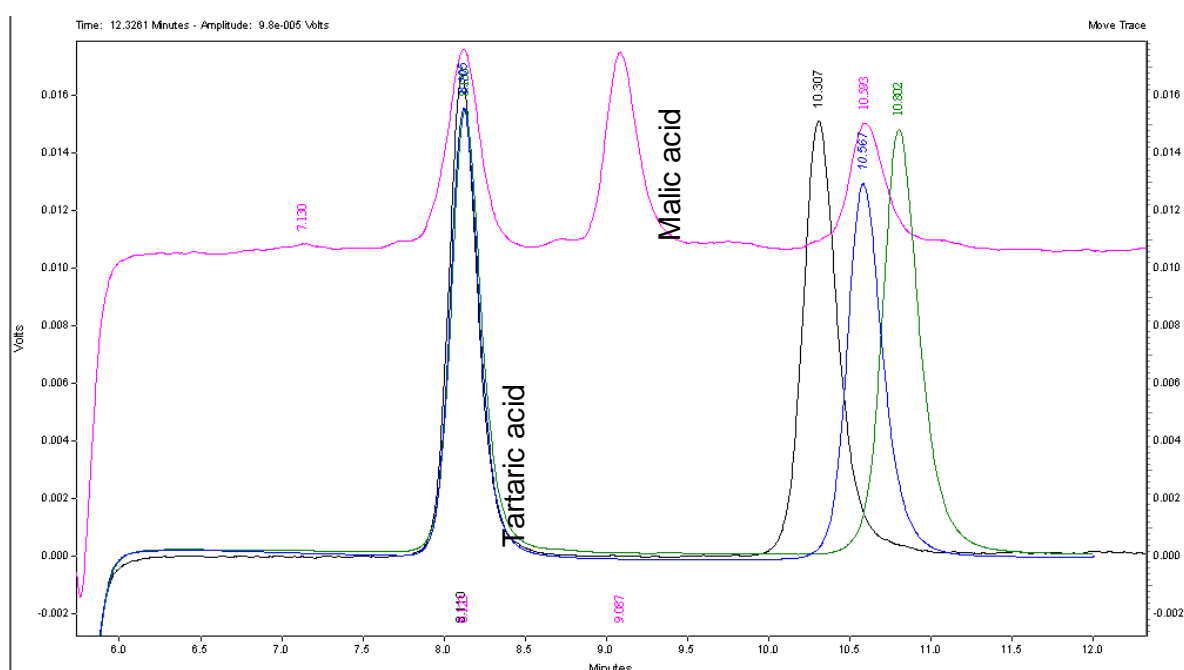


Figure 2.6 HPLC chromatograms of the supernatant of *M. pulcherrima* fermentation (pink), ribitol (black), arabitol (blue) and xylitol (green). All sugar solution concentrations are 1 g/L and all added tartaric acid as an internal standard. (Bio-rad Aninex HPX-87H 300x7.8mm column with H₂SO₄ 5 mM as an eluent at 0.6 mL/min)

Arabitol is a pentose sugar that yeasts in the *Candida clade* produce as a metabolic product and is believed to be part of the mechanism to preserve cell integrity from osmolytic pressure [88]. It can be used as diet-control sweetener as it is not metabolised by humans, like its more popular enantiomer; xylitol. Arabitol is presented in L-form and D-form as L-arabitol and D-arabitol which can be both produced by microorganisms. Most L-arabitol produced by microorganisms has arabinose as precursor while D-arabitol

is produced using glucose [89-91]. In contrast, most L-arabitol has been reported from the fermentation of L-arabinose [92-94]. Therefore, it is assumed arabitol in this work to be D-arabitol because it was fermented from glucose. Several yeasts are reported to produce D-arabitol from glucose, mostly osmophilic yeast, such as *Zygosaccharomyces rouxii*, *Debaryomyces hanseii*, *Candida albicans*, *Candida pelliculosa*, *Candida famata* and *Pichia miso* [95]. Koganti, *et al.* has reported arabitol production from several strains of *Metschnikowia zobellii* [96], though to date no reports have shown arabitol production from *M. pulcherrima*.

D-Arabitol is known to be produced by a few yeasts through glucose metabolism via pentose phosphate pathway (PPP). Its production in yeasts was investigated in osmophilic yeasts since the 1960s but did not receive much attention until the 1990s when interest in potential biomolecule building blocks were revived due to the need for non-fossil fuel based bio-products [88, 89]. The potential of arabitol in a biorefinery was recognised in 2004 when it was listed in the top 12 valued chemical building-block compounds from biomass by US Department of Energy [97]. According to the study, arabitol, grouped with its enantiomer xylitol, can be the building block chemical for the direct use as non-nutritive sweeteners and unsaturated polyester resins (UPRs). This suggesting that arabitol has potential to be another valuable product from *M. pulcherrima*, alongside the fragrance 2-phenylethanol.

2.4 Optimisation of *M. pulcherrima* Fermentation

Seemingly *M. pulcherrima* has the potential to produce 2PE and arabitol effectively and could be used as a basis for a bio-refinery. The optimal conditions for production were therefore assessed. The conditions studied included pH, temperature and nutrient (nitrogen) content in both SGJ and MSGJ.

Initially the effect of pH was studied where SGJ media was adjusted to pH 2, 3, 4 and pH 5. Tartaric acid is present in SGJ media and acted as a pH buffer. With these buffers, all experimental pH did not deviate more than $\text{pH} \pm 0.2$ from the starting point.

The biomass production and major product concentrations are given in Figure 2.7. *M. pulcherrima* is known to survive at low pH [24], however, at pH 2, the growth rate was very low, while some growth was observed after 24 hours, all growth has ceased by 72 hours and decreased after this point, potentially due to cell death. At first both fructose and glucose were consumed at a similar rate though after five days no further sugars were metabolised at this pH supporting the cell death theory. At higher pH, the amount of biomass was increased substantially with the optimal being pH 5. The yeast hit stationary phase after approximately 72 hours though this did depend on the pH. The growth rate of *M. pulcherrima* was the highest at pH 4 and 5 with a maximum growth rate at pH 4 of 1.044 g/g d^{-1} and 1.033 g/g d^{-1} for pH 5. This was not only a reasonable rate for biomass production but excitingly pH 4 would help ward off invasive species, while still guaranteeing high productivity from the yeast.

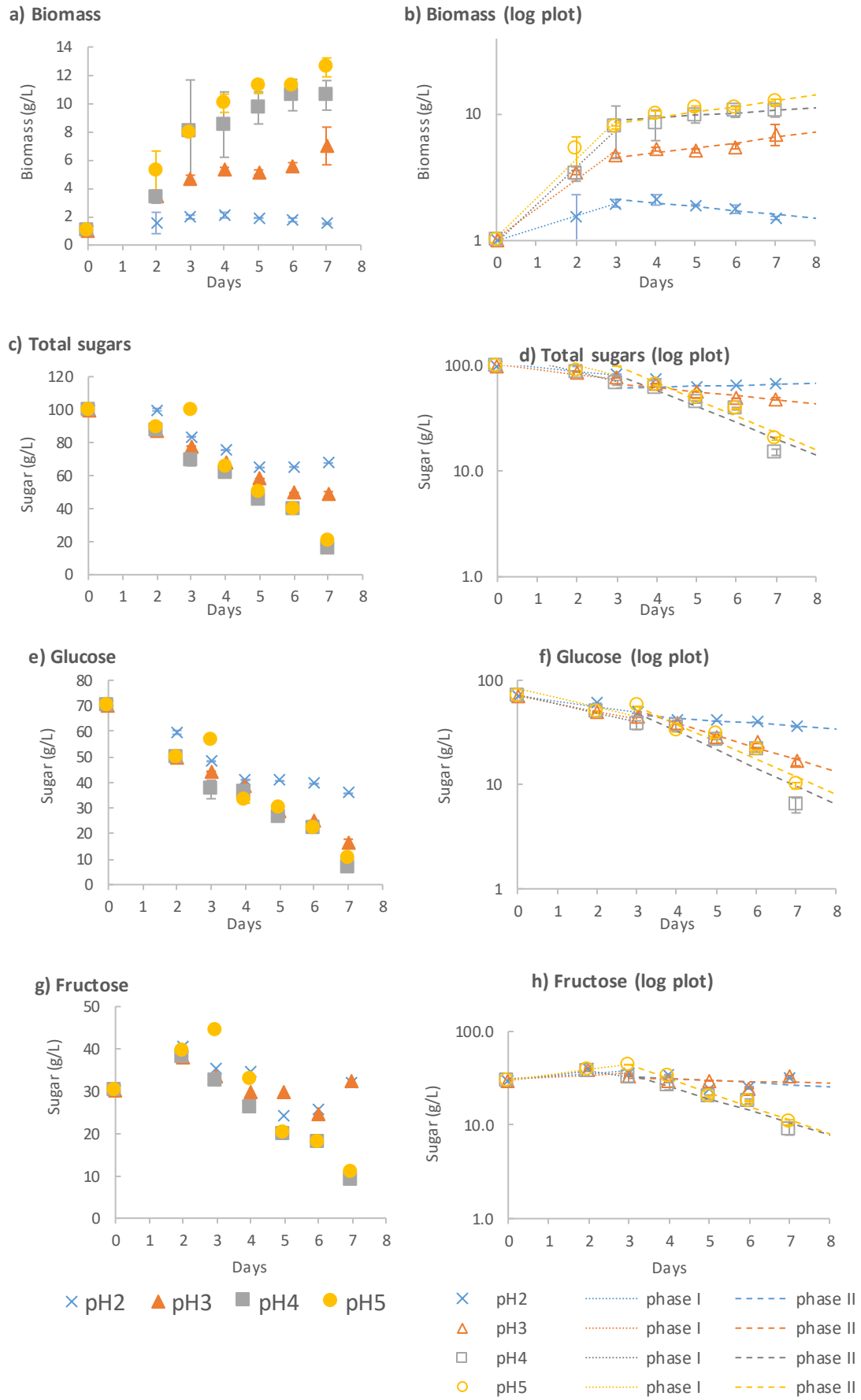


Figure 2.7 Growth and sugar utilisation profiles of *M. pulcherrima* cultured in SGJ media at 20°C and pH 2, 3, 4 and 5. (n=3, error bars represent one s.d.)

Hexose sugars such as glucose, fructose and mannose are the common and preferred carbon source for most yeast species, and it is clear that *M. pulcherrima* is well adapted to using them [98, 99]. The metabolism of glucose in yeast can be in at least two well-known pathways. The most well-known glucose utilisation pathways are glycolysis, responsible for 66-80% of glucose metabolism in most cells, and the other is the pentose phosphate pathway (PPP) which is usually responsible for the rest of the metabolism, though some other pathways can be found in other organisms. [100]. Cells generate most of their necessary energy and essential chemicals as amino acids, proteins, nucleic acids etc. for cell growth and other activities. Fructose is also a favourable carbon source for yeast as well, however, the cellular fructose utilisation pathway, or fructolysis, as opposed to glycolysis in glucose metabolism, is different but connected to the main glycolysis pathway.

Glucose and fructose utilisation rate in the yeast *Saccharomyces cerevisiae* was shown by [101] that glucose and fructose are utilised by the yeast at a similar rate in the separated experiments. However, when the yeast co-ferment glucose and fructose, the sugar uptake rate is suppressed by about 30-50% in the glucose 100 g/L with fructose 10 g/L fermentation and the rate of fructose uptake is even more affected by the presence of glucose. It is likely that the rate of sugar uptake in *M. pulcherrima* fermentation is similar and the uptake rate is reduced by the co-fermentation of glucose and fructose.

The production of 2PE, ethanol and arabitol was assessed as a function of pH (Figure 2.8). At pH 2, no ethanol or 2PE were detected in the culture though interestingly up to 9 g/L of arabitol was produced. This cessation of activity is presumably due to the high toxicity of the low pH, and its effect on the general cell health.

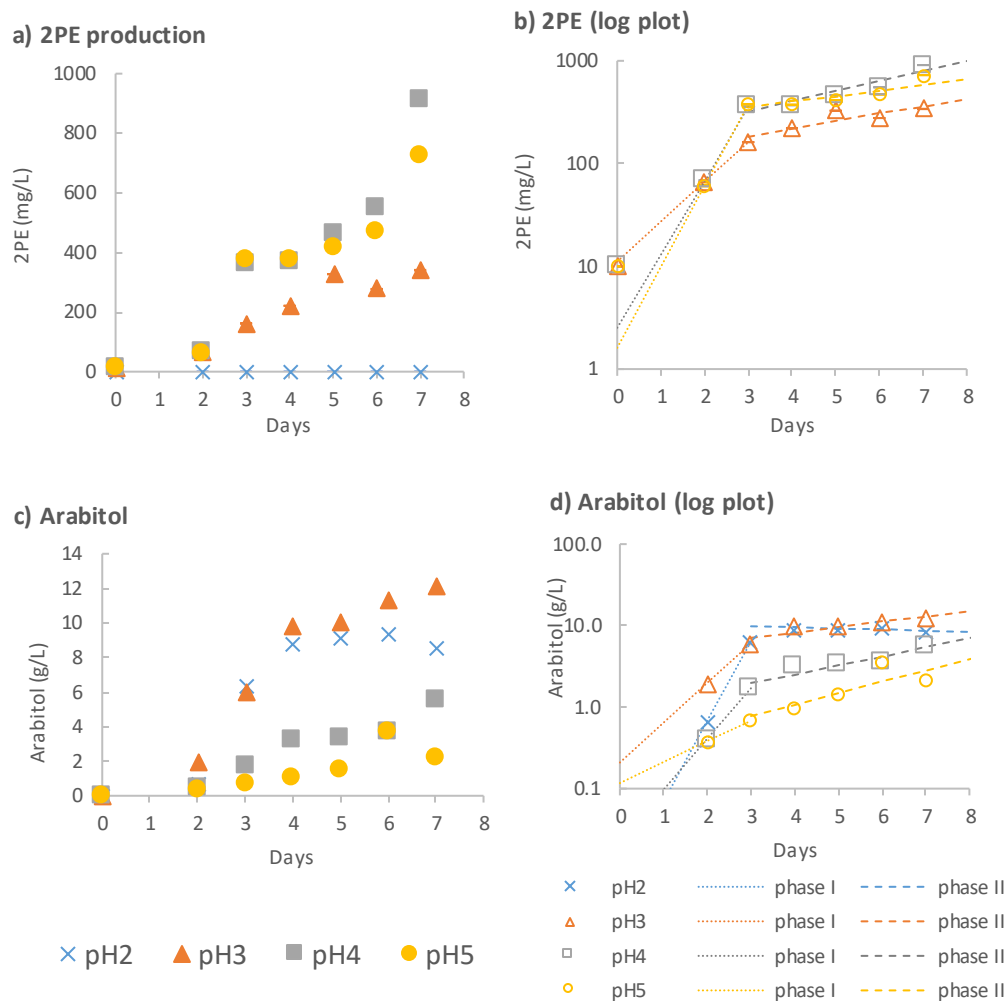


Figure 2.8 Product profiles of *M. pulcherrima* cultured on SGJ media at pH 2, 3, 4 and 5 at 20°C. (n=3, error bars represent one s.d.)

More biomass was produced at pH 3, which coincided with the highest arabitol production of 12.5 g/L. Arabitol production then decreased with increasing pH, with *M. pulcherrima* producing 6 g/L at pH 4 and only 2 g/L at pH5. Arabitol seems to be produced mostly in the growth phase with only a slight increase observed in the stationary phase.

While the optimum conditions for biomass production were pH 5 the optimal condition for 2-PE was found to be pH4. Almost 1 g L⁻¹ was produced by *M. pulcherrima* at this pH, this is far higher than most yeasts recorded in the literature and 2 magnitudes higher than *M. pulcherrima* is known to produce in the wine fermentation. However, this means that while arabitol can also be produced from *M. pulcherrima*, the optimal ranges for production do not overlap.

Due to the high growth rate at low pH, the culture would have a competitive advantage against invasive species and the high 2PE production, pH 4 is seemingly the optimal pH for 2PE production. It was shown by [24] that *M. pulcherrima* cultured at pH 4 can

maintain an axenic culture in the non-sterile condition. Ethanol is also produced in the exponential growth phase by *M. pulcherrima*, especially at the early stages when the yeast has presumably used up most oxygen for metabolism. Ethanol can act as an inhibitor at high concentrations. This could potentially inhibit alternative product formation as well as growth. The toxicity of ethanol including its synergistic effect with 2PE was investigated later.

2.4.1 Effect of temperature

Temperature is also an important environmental condition in fermentation. The strain used in these experiments has demonstrated a wide optimal temperature range in the literature [24]. To determine the effect of temperature on product formation further experiments were undertaken at 15 °C and 28 °C at pH 4. The biomass and product formation in the *M. pulcherrima* fermentation at 15 °C, 20 °C and 28 °C are shown in Figure 2.9.

At the colder temperature at 15 °C, the yeast was found to be less active, with a lag phase of 2 days and grew more slowly than at 20 °C but by the end of the fermentation a similar amount of biomass was observed as was at 20 °C.

At the higher temperature (28 °C), it was expected that *M. pulcherrima* would demonstrate higher growth as other yeasts such as *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* usually have the warmer optimum temperature of 28-40°C [102-104]. However, *M. pulcherrima*'s growth in SGJ media was halted at high temperature. The yeast reached the log growth phase for a while but stopped growing after day 3. After that the cell mass was steady and decreased slightly. The maximum biomass at 28°C achieved only 2 g/L after day 3 compared to 10g/L at 20°C. This demonstrates that the strain has adapted to the lower temperatures observed in the UK, and is highly suitable for processing without the need for additional heating.

The rate of sugar metabolism is lower at 15 °C than at 20 °C, which can be seen from log-plotted sugars in Figure 2.9, though, at both these temperatures sugar was utilised constantly throughout the experiment in both the growth and stationary phases. In contrast, at 28°C after day 3 no further sugar was used, though the sugar was initially utilised by the yeast at the higher rate in the first few days. This strongly indicates the dying phase in the high temperature. However, arabinol production was observed after the dying phase in 28 °C with no signs of substrate utilisation. This could possibly resulted from the recycle of carbon from dying cell and enzymes which involved in arabinol production may still active even when the cells are dying.

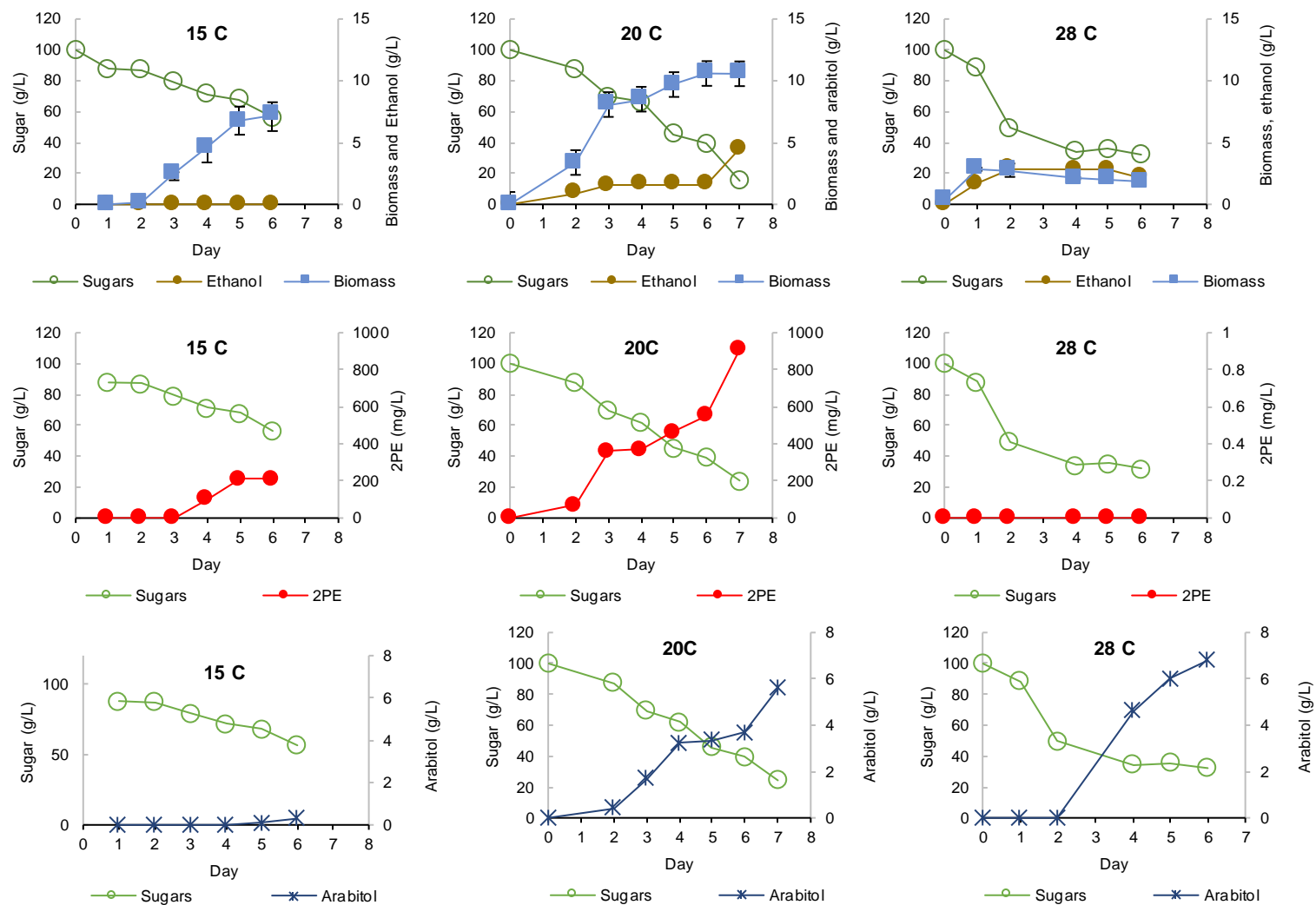


Figure 2.9 Fermentation profiles from *M. pulcherrima* culture in shake flasks in SGJ media pH4 at 15 °C, 20 °C and 28 °C. (n=3, error bars represent one s.d.)

The metabolic product 2PE was produced during the log growth phase only. In contrast, arabitol was not restricted to log growth phase, and the yeast kept on producing it if there was substrate available. Ethanol was also observed, seemingly the product from anaerobic metabolism during the oxygen deficiency in the log growth phase due to high growth and oxygen demands.

The fermentation products were not produced in high quantities at the low temperature. For example, arabitol and ethanol were barely detectable at 15 °C. Potentially due to the lower growth rate, the yeast is less productive and a high DO is maintained, reducing ethanol production. The highest level of arabitol was produced at 28 °C. While the growth of the yeast had stopped and decreased since day 3, the production of arabitol continued to increase until the end of the experiment.

Arabitol production is generally related to cell growth [95]. While the growth at 15 °C was slowed down by the colder temperature but was not affected by the highest biomass, it seems to limit the arabitol production in the growth period. This could be either advantageous, depending on the purpose of fermentation. If arabitol production is not needed, the colder fermentation temperature will reduce the need to separate arabitol from unwanted products.

Ethanol production during the fermentation was highly affected by the temperature of the fermentation. The highest ethanol production was observed at the higher temperatures. At 28°C, the ethanol production was produced during the first 3 days at double the rate of the culture at 20°C suggested the high aerobic activity that used up oxygen and created the anaerobic condition which resulted in the production of alcohol. The increased temperature increased the ethanol production rate as well. But since day 3 where the growth of the yeast stopped and the growth curve was similar to the ethanol production. It is possible that higher-than-usual ethanol level can be toxic to the yeast and is the growth limiting factor to the yeast.

Even at 15 °C 2PE is still produced though to a lesser extent than at 20°C, demonstrating the complexity of the two pathways used to produce 2PE from glucose, and the temperature dependence of them. The optimal temperature for overall product formation was confirmed to be 20°C where the culture yielded 10 g/L biomass, and 980 mg/L 2PE, with an acceptable production of arabitol.

2.4.2 Effect of glucose concentration on the production of 2PE

Up to this point a relatively high sugar loading (100 g/L) was used. However, sugar loading can have a large effect on the formation of products due to metabolic and osmotic stresses. To understand how sugar concentration affects the 2PE production in *M. pulcherrima*, the yeast was cultured in SGJ media at 20°C and pH4. Glucose was used as the carbon source. The glucose concentrations examined were 25, 50, 75 and 100 g/L. Sugar loadings above 100 g/L are known to negatively affect the fermentation, and so weren't examined. The C/N ratio was kept constant for all the media used, to negate the effect of nutrient depletion at lower carbon loadings.

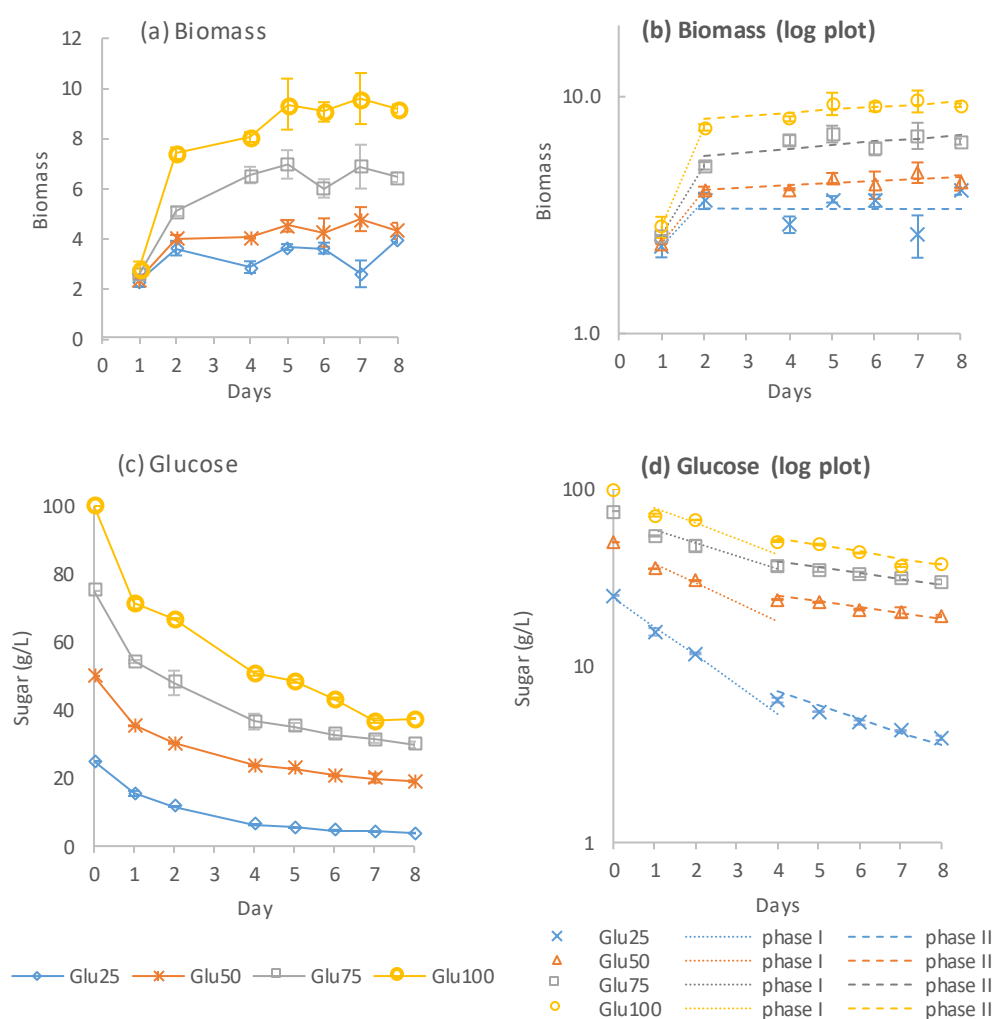


Figure 2.10 Growth and sugar utilisation of *M. pulcherrima* grew in SGJ media with 25, 50, 75 and 100 g/L glucose concentration. ($n=3$, error bars represent one s.d.)

The growth of *M. pulcherrima* under different carbon loadings is given in Figure 2.10. The lower the glucose concentration, the lower the growth, with the highest yeast biomass achieved with 100 g/L of glucose. This demonstrates that there is no osmotic

inhibition, and that under these conditions *M. pulcherrima* could be described as an osmophilic yeast. This is potentially beneficial as being osmophilic means that the yeast is less prone to contamination and could grow in non-sterile conditions, at high loadings of sugar that reduces the volume of the bioreactors needed.

In SGJ media the rate of glucose assimilation is similar at every concentration of glucose, though at 25 g/L it was slightly higher.

The concentration of glucose has a large effect on the 2PE and arabitol production of *M. pulcherrima* (Figure 2.11). 2PE production from *M. pulcherrima* is highest in SGJ media with glucose at 100g/L, while some 2PE is observed at lower sugar loadings, none was observed at a glucose loading of 25 g/L.

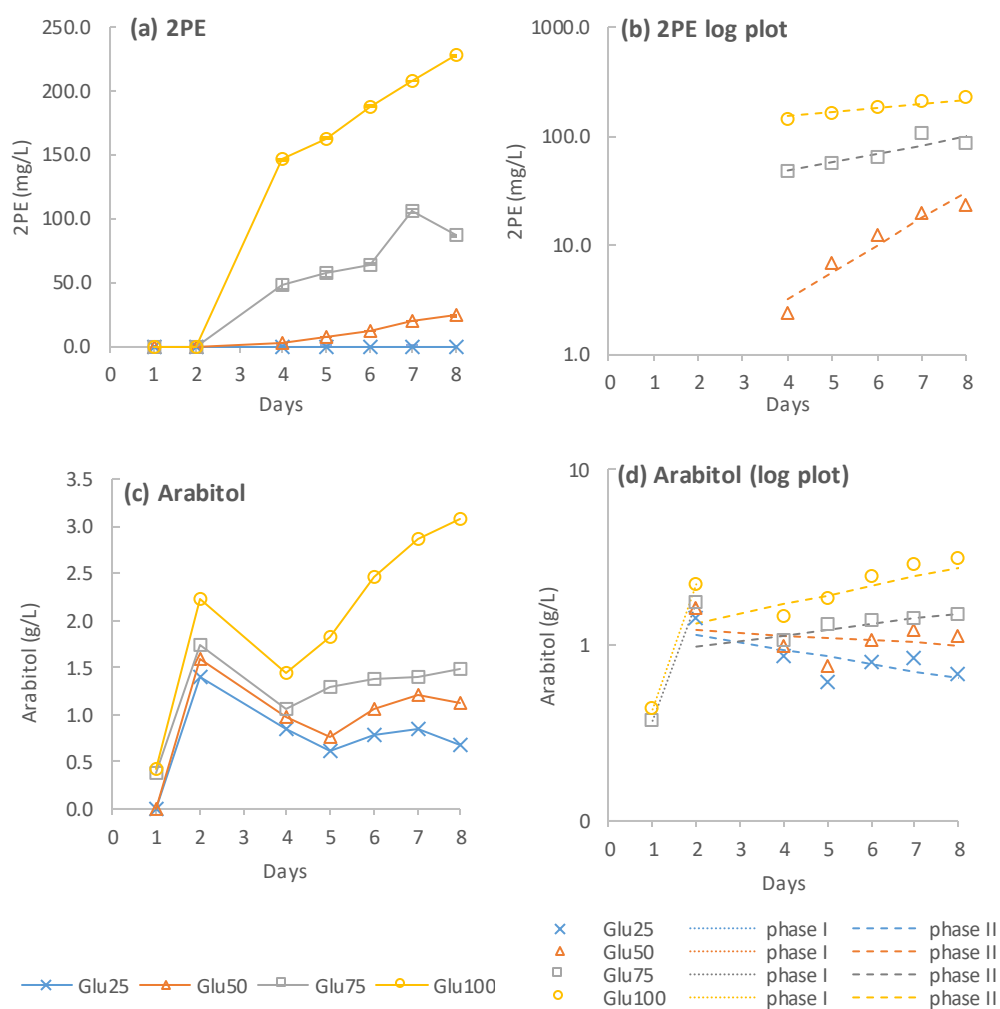


Figure 2.11 Production of 2PE and arabitol from *M. pulcherrima* culture in SGJ media with 25, 50, 75 and 100 g/L glucose concentration

2PE production was observed at the end of exponential growth phase and increased slightly during the stationary phase. The rate of production of 2PE is different at the

different loadings. The rate of 2PE production was higher at the lower glucose concentration. However, because t

High 2PE yields are afforded at high glucose loadings presumably as the amino acid precursors, such as L-phenylalanine, are produced via the shikimate pathway, which needs high glucose loadings to trigger the carbon flux through the pathway [105].

Arabitol production is also closely linked to glucose concentration, with the highest yields being afforded under high glucose conditions. This suggests that *M. pulcherrima* is not using arabitol to change the osmotic pressure of the media and rather might be using it as a store of niche carbon outside of the cell. The reduction in the total arabitol yield at day 4 does suggest the yeast can re-metabolise the C₆ sugar.

2.4.3 Effect of nitrogen concentration on 2PE production

2PE is produced via the Ehrlich pathway from L-phenylalanine as a precursor which, in the yeast cell, can be synthesised via the aromatic amino acids in the Shikimate pathway. However, it was seen that in the early experiments with MSGJ media that 2PE production is lower than in the SGJ media which has lower nitrogen available. Therefore, the effect of nitrogen concentrations on the production of 2PE was investigated (Figure 2.12)

M. pulcherrima was cultured in SGJ media with $\text{NH}_4(\text{HPO}_3)_2$ as the sole nitrogen source at different concentrations, (0.1, 0.2, 0.5 and 1 g N/ 100 g carbon). The SGJ media contained glucose at 100 g/L as substrates. The end products were analysed at the end of fermentation at 7 days.

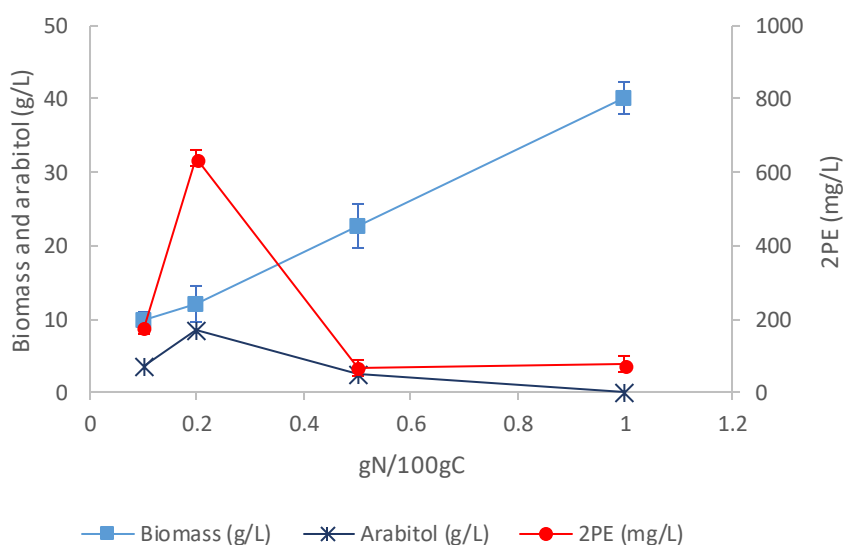


Figure 2.12 Effect of N/C ratio on growth and production of 2PE and in SGJ media at 20°C, pH4 (n=3, error bars are given as one s.d.)

When the nitrogen is less than 0.5 g N/100 g C, the biomass production was limited by the limited nitrogen. In the condition that the nitrogen is at least 0.5 g N/100 g C, the biomass reached the maximum growth without a growth limiting effect. Increasing nitrogen from 0.5 to 1 g N/100g C did not increase the production of biomass as much as from 0.1 to 0.2 or from 0.2 to 0.5 g N/100g C. However, 2PE is produced highest at 0.2 g N/100 g C, yielded approximately 500 mg/L 2PE. When the nitrogen is less than 0.2 g N/100 g C, the yeast seems to be affected by the limited growth and affected other metabolites production such as 2PE as well.

However, when nitrogen was increased to 0.5 g N/100 g C, 2PE production decreased dramatically, and was finally lowest at 1g N/100g C. This may be because in the limited nitrogen source and abundant carbon source, the yeast growth is stimulated and require amino acids for building new cell, which might have diverted amino acid synthesis production towards necessary proteins. *M. pulcherrima* might be preferred to produce L-phenylalanine to other amino acids, which is partially converted to 2PE via the Ehrlich Pathway. But when the condition of growth has abundant nitrogen, L-phenylalanine production via Shikimate pathway is not necessary, leading to less L-phenylalanine production and, consequently, less 2PE production.

2.5 Cytotoxicity: synergistic toxicity of 2-phenylethanol and ethanol to *M. pulcherrima*

M. pulcherrima produces several metabolites, such as 2PE and ethanol, during the fermentation which can be toxic in high loadings. Even though 2PE is the valuable compound that we are trying to increase the production as high as possible, it is a known antimicrobial compound which could inhibit growth and also 2PE production itself. [106, 107] Ethanol also plays important role in yeast fermentation by its toxicity, for example at the end of wine fermentation. As inhibitors can have a synergistic effect, 2PE toxicity was examined in conjunction with ethanol.

The experiment was conducted in 96-well plates using SGJ media with the mixture of ethanol (0-12 %v/v) and 2PE from 0-2 g/L (n=6). The experiment was run at 20°C and pH4.

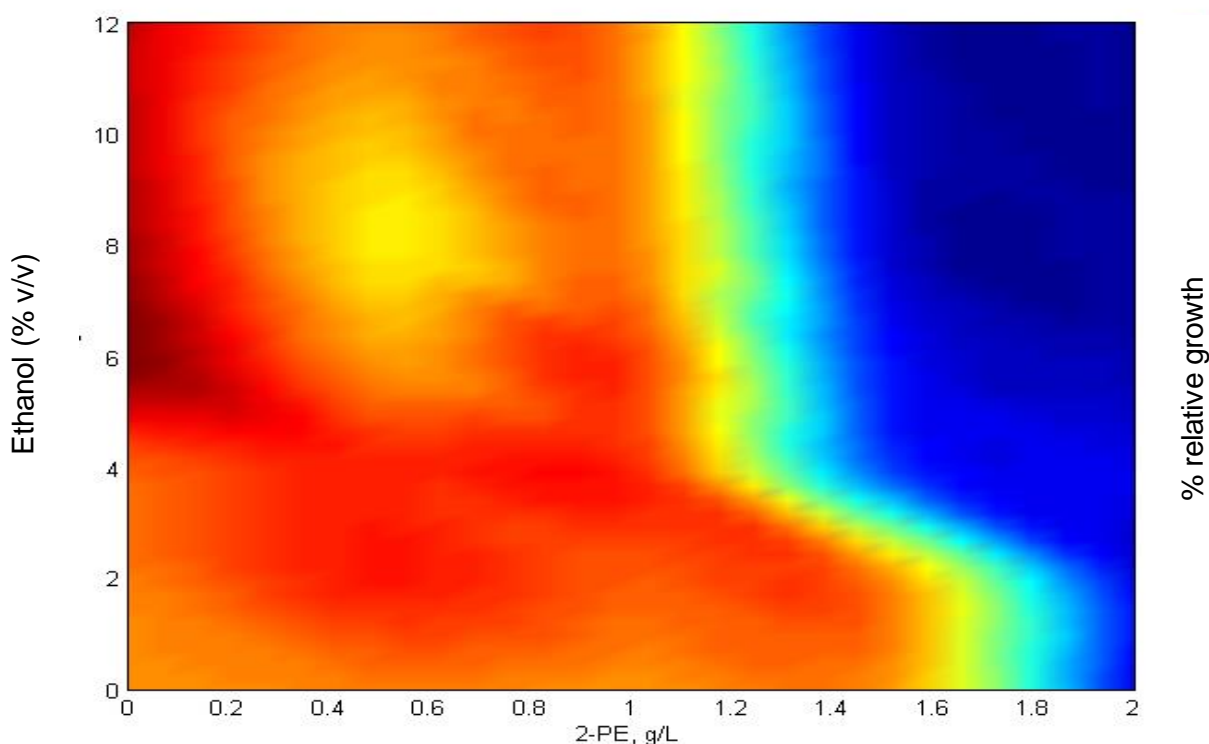


Figure 2.13 Synergistic toxicity of 2-phenylethanol and ethanol to *M. pulcherrima* in 96-well plates at 20 °C at pH 4. ($n=6$) (red = best growth, blue = worst growth)

In Figure 2.13, the red zone demonstrates the best growth whereas the blue zone shows limited growth. With no ethanol, *M. pulcherrima* can tolerate 2PE up to approximately 1.5 g/L where it started to show signs of inhibitory effect to the growth, by 1.8 g/L the growth was reduced to just 20% compared to the control.

When ethanol was added up to 2% v/v, it did not affect 2PE tolerance of *M. pulcherrima* though higher concentrations started to demonstrate reduced growth. Combined with 4% ethanol, the yeast's tolerance to 2PE is reduced to approximately 1.1 g/L. Above 4% the increased ethanol concentration did not have a further inhibitory effect. In the previous optimisation at temperature 28°C, it was possible that ethanol was one of the inhibitors limiting growth and reducing 2PE production. However, these results suggest that ethanol is not unduly toxic to *M. pulcherrima* even in combination with 2PE.

Arguably, this study was done in the 96-wells plate (working volume 195 μ L/well) and the results may differ in a larger fermentation. However, it did show that this strain shows a relatively high ethanol tolerance. In the condition that there was no 2PE, *M. pulcherrima* could tolerate ethanol up to 12 %v/v without any sign of inhibition.

2.6 Effect of strain variation on the product formation

M. pulcherrima seems a promising strain for 2PE and arabitol production especially under high sugar loadings at high C/N ratios. However, these preliminary results are based on one strain, to examine the variation from strain to strain, four strains isolated from the surrounding countryside were studied. The four strains of *M. pulcherrima* were named strain A1, A35, B6 and G6.

All the strains were grown in SGJ media, and were grown in triplicate in 125 mL shaking flasks. Figure 2.14 shows the cultures at the end of fermentation at day 15, from left to right, strain A1, A35, B6 and G6. While strain A1 and G6 have a yellowish colour, similar to the colour of the media, the culture A35 and B6 had a purple-red tinge in the culture, this is presumably due to the ability of *M. pulcherrima* to produce pulcherrimin, the iron organo-metallic complex [108], the lack of pulcherrimin in some cultures is not uncommon [109], and the yeast does demonstrate large strain-strain variation.



Figure 2.14 Different strains of *M. pulcherrima* fermentation at the end of experiment at day 15, from left to right, A1, A35, B6, G6 (n=3)

All 4 strains of *M. pulcherrima* grow well in SGJ even though the C/N ratio is low (100:0.18). After 15 days, all 4 strains showed the same detectable compounds such as arabitol, 2PE, ethanol (Figure 2.15).

There is a large strain to strain variability in the amount of biomass produced. The strain G6 generated the maximum biomass, slightly better than strain A1 with over 10 g/L of biomass observed in these cultures. The lowest biomass produced from this group was

from strain B6, yielding only 4.6 g/L compared to 15.6 g/L and 12.6 g/L from strain G6 and A1 respectively.

All the strains produced ethanol at approximately similar low concentrations. Arabitol is also produced by all 4 strains but significant strain to strain variation was observed. Two strains, A35 and B6, produced over 5 g/L where less than half this was produced by A1 and G6. Interestingly, both A35 and B6 were the strains that produced pulcherrimin (the purple-red pigment). This suggests that there is some relationship between arabitol and pulcherrimin production.

There was also significant per cell variation in the amount of 2PE produced. Strain B6, that produced the lowest biomass yielded the highest production of 2PE. 2PE production from all strains are in the range of 320-520 mg/L, some of the highest production levels of any yeast [110, 111]

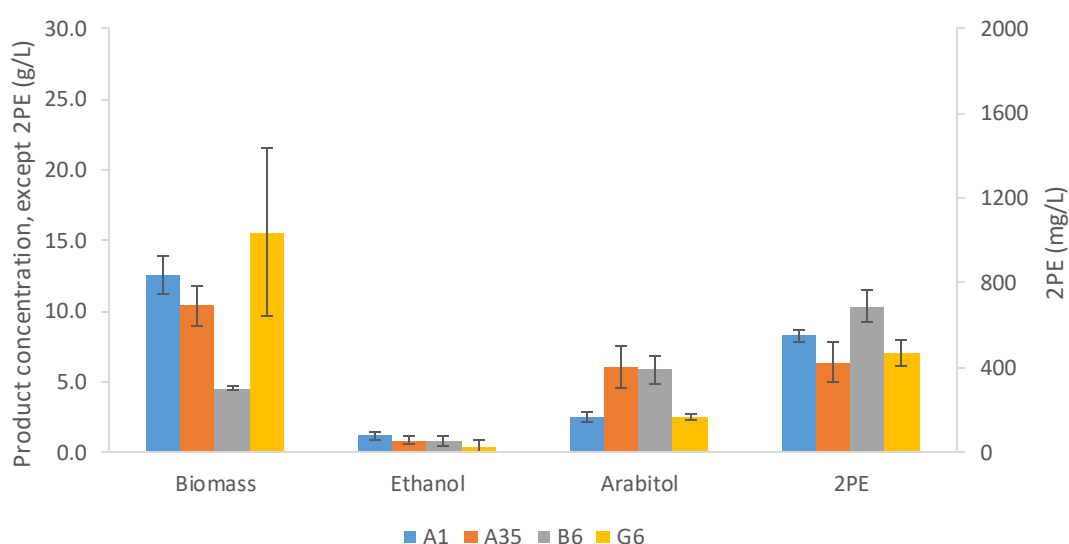


Figure 2.15 Fermentation products from 4 strains of *M. pulcherrima* in synthetic grape juice (SGJ) media after 15 days in SGJ media pH4 at 20°C (n=3, error bars represent one s.d.)

The fermentation profiles of *M. pulcherrima*, showing the production of yeast biomass, 2PE, arabitol and sugar utilisation from the 4 strains is given in Figure 2.16.

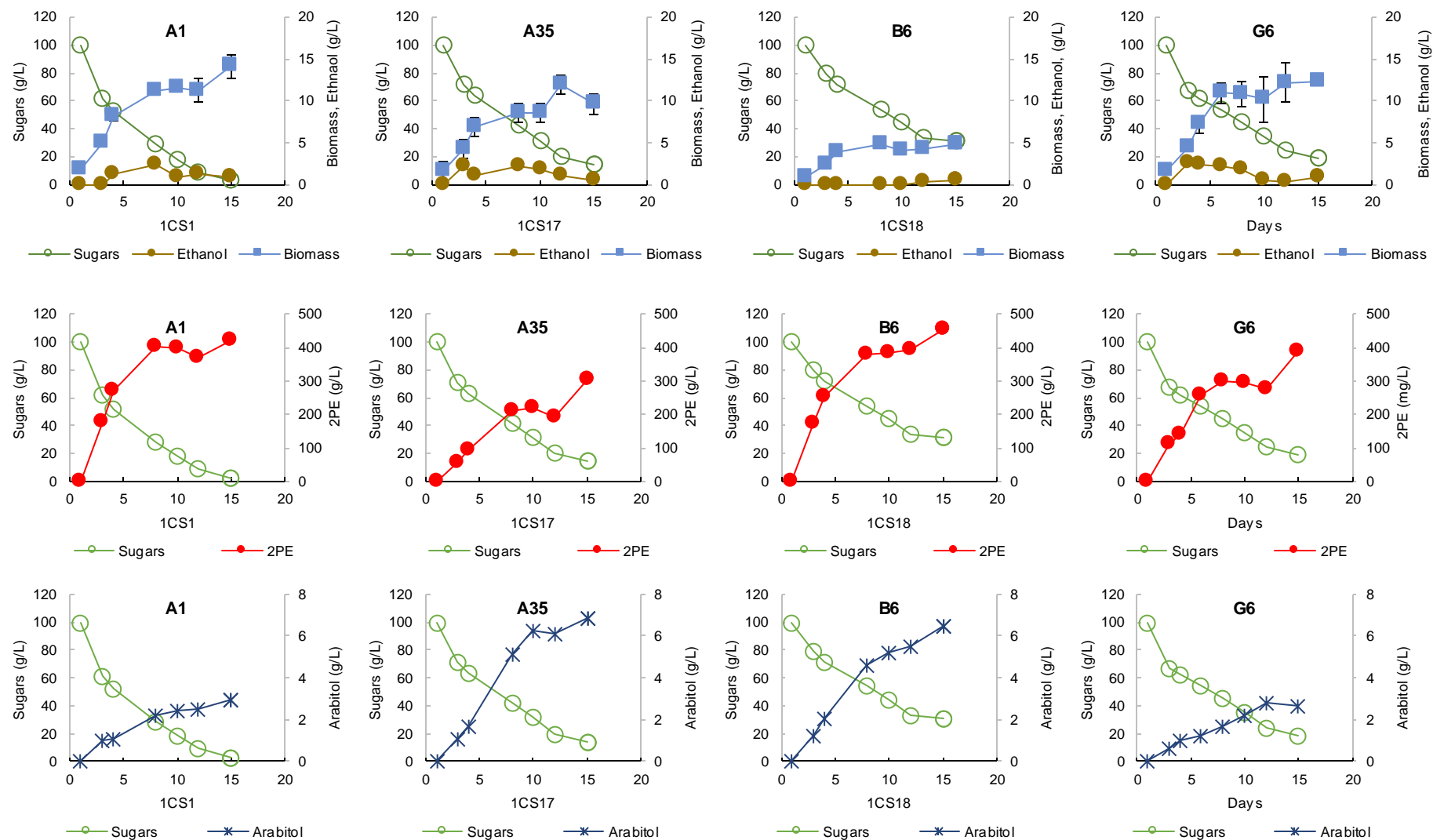


Figure 2.16 Fermentation profiles of *M. pulcherrima* strain A1, A35, B6 and G6 in SGJ media pH 4 at 20°C ($n=3$, error bars represent one s.d.)

The products formed and sugar utilisation in the fermentation was similar for all strains tested. For example, all of the strains have a short lag time and reach the stationary phase after approximately 3-4 days. Among the 4 strains, A1 and A35 demonstrate the fastest growth rate and achieved the highest biomass. All strains had grown in the exponential phase from day 1 till day 5. After that the growth slowed with all strains reaching the stationary phase at approximately the same time, even though the rate and the final biomass concentration were different. The growth rate of *M. pulcherrima* in the growth phase is in the range of 0.24 to 0.38 g/g. day⁻¹, where strain G6 and A1 are the highest and strain B6 is the lowest (Figure 2.17)

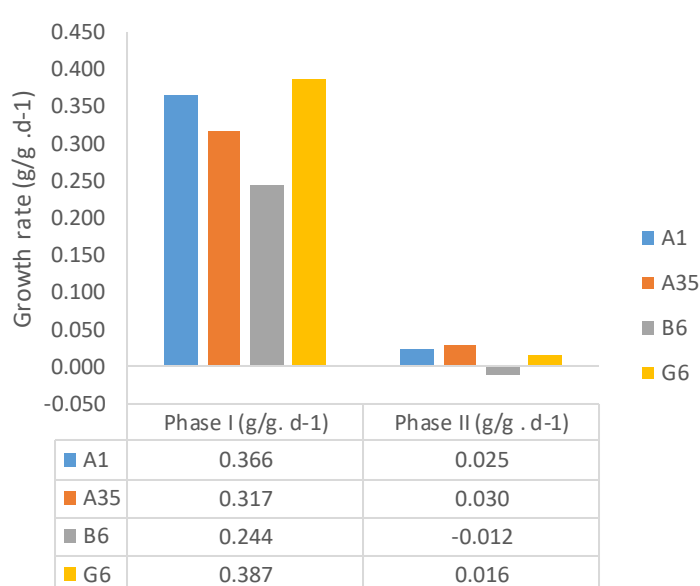


Figure 2.17 Growth rate of *M. pulcherrima* strain A1, A35, B6 and G6. Phase I designated as growth phase and phase II as stationary phase

During the exponential growth phase, all the yeast except B6 produced ethanol, though the production was low for all strains. The ethanol level rose rapidly to the maximum level around day 3, it is plausible that the cultures are oxygen limited at this point due to the rapid growth rate. When the growth is slower in day 4 and later, production of ethanol was slower as well and the yeast started to use ethanol as a substrate resulting in a reduced ethanol level. All strains that produced ethanol demonstrated a similar level of ethanol production and rate of ethanol assimilation.

B6 is the only strain that did not show any production of ethanol in the growth period. As it has the lowest growth among the strains, less than half of A1 and G6, this may be a function of the low biomass production that did not use up the oxygen which would trigger the ethanol production.

Compared to a general wine fermentation, the ethanol production for all the strains is fairly low. For example, Clemente-Jimenez *et al.* reported that *M. pulcherrima* can produce up to 5.4% v/v, in microvinification of Macabeo grape. Though the sugar loading was far higher and were cultured under oxygen limited conditions [112].

All the strains produced 2PE predominantly in the exponential phase. In the stationary phase, similar to the biomass, the 2PE remains fairly constant despite the sugar continuing to be metabolised, suggesting that it is being directed towards alternative products such as lipids [24].

The profile of arabitol production from *M. pulcherrima* strain A1, A35, B6 and G6 are markedly different (Figure 2.18) and could be separated into 2 groups, A35 and B6 which produced high arabitol, up to 7g/L, and A1 and G6 groups which produced lower arabitol, up to 3 g/L. The production of arabitol from *M. pulcherrima*, unlike the biomass and 2PE, was not limited solely to the exponential phase and is produced by all strains until day 10. The slower production after day 10 seems to be from sugar limitation rather than any other factor. For arabitol production, strain A35 and B6 seems to be the best candidate.

Finally, the sugar metabolism was examined for all four strains. All strains of *M. pulcherrima* can metabolise glucose and fructose with little difficulty, though the rate of glucose uptake was slightly higher than fructose. The log graphs demonstrate that sugar utilisation rate of A1 is the highest and of B6 is the lowest. But all strains share a similar trend. One interesting point is the rates did not change from the growth phase to the stationary phase. Even though the biomass and some other products such as 2PE were produced during the growth period and almost stopped in stationary phase, the yeast did not change the sugar utilisation activity and continue use in the same rate.

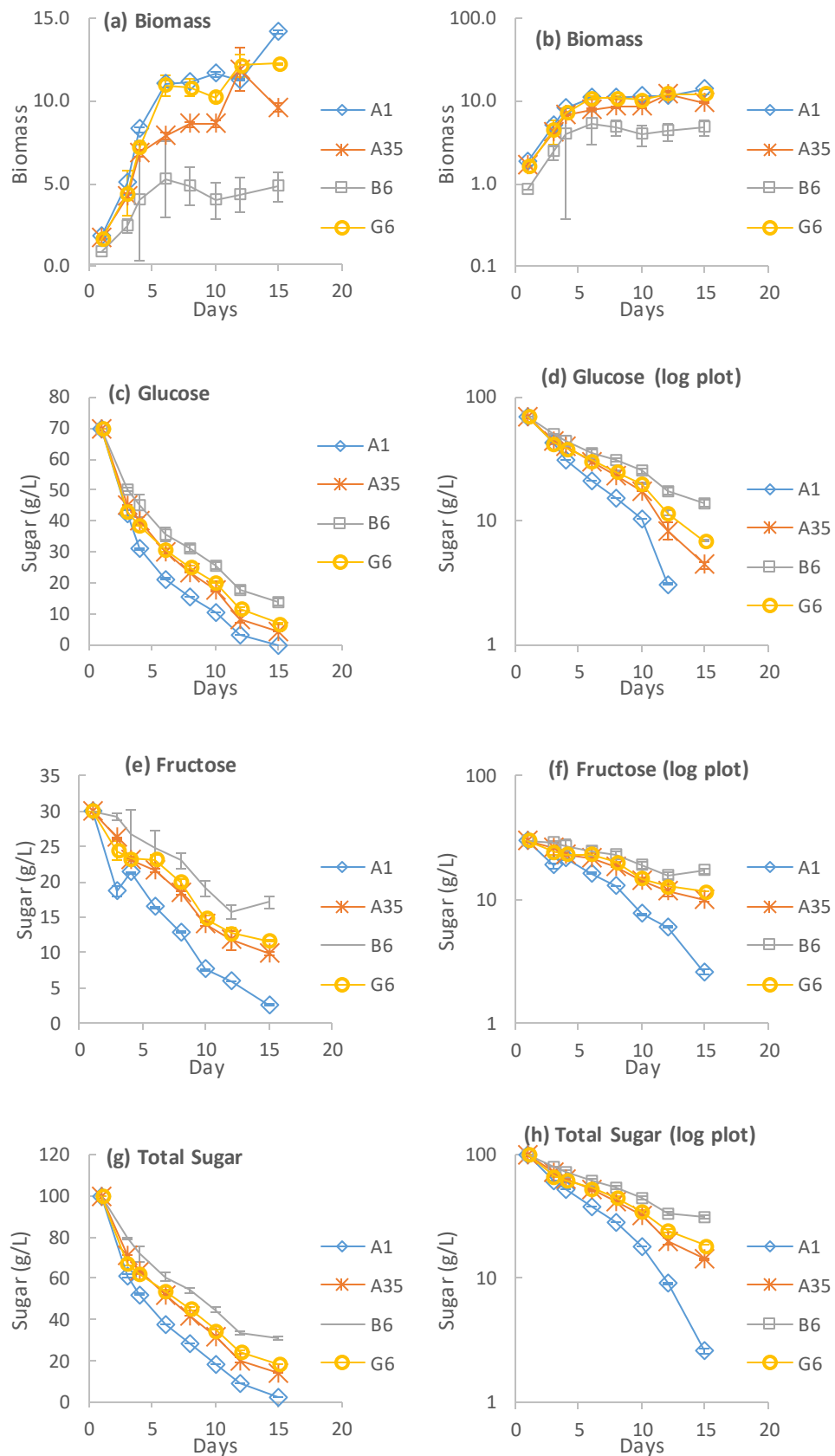


Figure 2.18 Profile of sugar utilisation during the fermentation of *M. pulcherrima* strain A1, A35, B6 and G6 in SGJ media ($n=3$, error bars represent one s.d.)

2.7 Alternative substrates

While these are promising results in the conversion of glucose to 2PE, glucose is relatively expensive and for larger scale production raises concern over competition with the food market. Two further sugar feedstocks, glycerol and xylose were therefore investigated to assess the possibility of growing *M. pulcherrima* from non-food substrates.

2.7.1 Xylose

Xylose is a pentose sugar (C₅) produced abundantly by plants. It is one of the sugars that makes up the complex hemicellulose fraction in lignocellulose. Both cellulose and hemicellulose are part of the plants cell wall matrix structure. While cellulose is composed of glucose, and tends to be strong, crystalline and resistant to hydrolysis, hemicellulose is random, amorphous and relatively easier to hydrolyse. Xylose can represent up to 34% in lignocellulosic material from plants. But due to the complex structure of lignocellulose and resistance to hydrolysis, glucose and xylose in cellulose and hemicellulose structure are not readily available for most microbial fermentations and need to be hydrolysed before.

The metabolism of xylose is different from glucose and will not always yield the similar products as a glucose fermentation. As in the well-documented study of ethanol production, for example, wild *Saccharomyces cerevisiae* can ferment ethanol from glucose but, while it can assimilate xylose, it cannot directly produce ethanol from xylose. The pathway also differs from each species. Several yeasts have been reported to be able to ferment xylose to ethanol such as those in genera *Brettanomyces*, *Candida*, *Clavispora*, *Kluyveromyces*, *Pachysolen*, *Pichia* and *Schizosaccharomyces*. In this study, the suitability of using xylose for 2PE production was assessed.

Sugar fermentability by *M. pulcherrima* was studied by culturing *M. pulcherrima* in SGJ media with four monosaccharide sugars (glucose, xylose, cellobiose and arabinose) with 7.5 g/L each and with adequate nitrogen available (C/N 100:1). The fermentation was conducted in regular condition for *M. pulcherrima* at pH4 and 20°C.

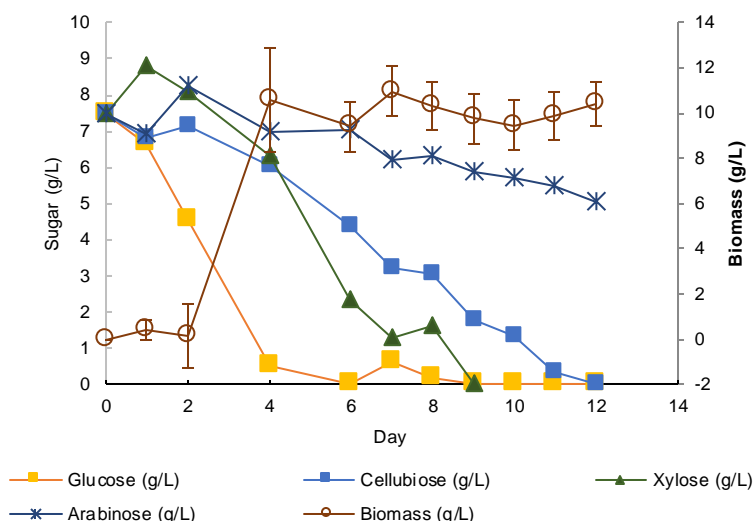


Figure 2.19 Glucose, xylose, cellubiose and arabinose fermentability by *M. pulcherrima* in media contained each sugar 7.5 g/L at 20°C and pH4 (n=3, error bars represent one s.d.)

In Figure 2.19, glucose, among these four sugars, is readily consumed by *M. pulcherrima* followed by xylose, cellubiose and arabinose, respectively. The yeast grew regularly to a maximum growth in 4 days and the glucose was almost used up. The yielded biomass is relatively high, not different from the culture with sole glucose as carbon source with a similar sugar concentration (30 g/L), possibly due to the availability of nitrogen. When glucose was still left, other sugars were not consumed, suggesting that glucose is the preferred carbon source. Xylose and cellubiose utilisation did not occur until no glucose was left. Both xylose and cellubiose were used up at a similar rate, though arabinose was used the least and not completely utilised even late in the fermentation when no other sugars were available. Pitt and Miller (1968) studied several properties of *M. pulcherrima* including sugar assimilation. In this work, they reported that *M. pulcherrima* was able to use glucose, xylose and cellubiose but couldn't metabolise arabinose [7]. This is a definite advantage of using *M. pulcherrima* over *S. cerevisiae* which cannot metabolise xylose.

Further study for xylose fermentation and its metabolic products from *M. pulcherrima* was conducted with the modified xylose grape juice (MXGJ) media which was identical to modified synthetic grape juice (MSGJ) media except xylose 30g/L was used instead of fructose of the same concentration with relatively high nitrogen at C/N ratio 100:1.

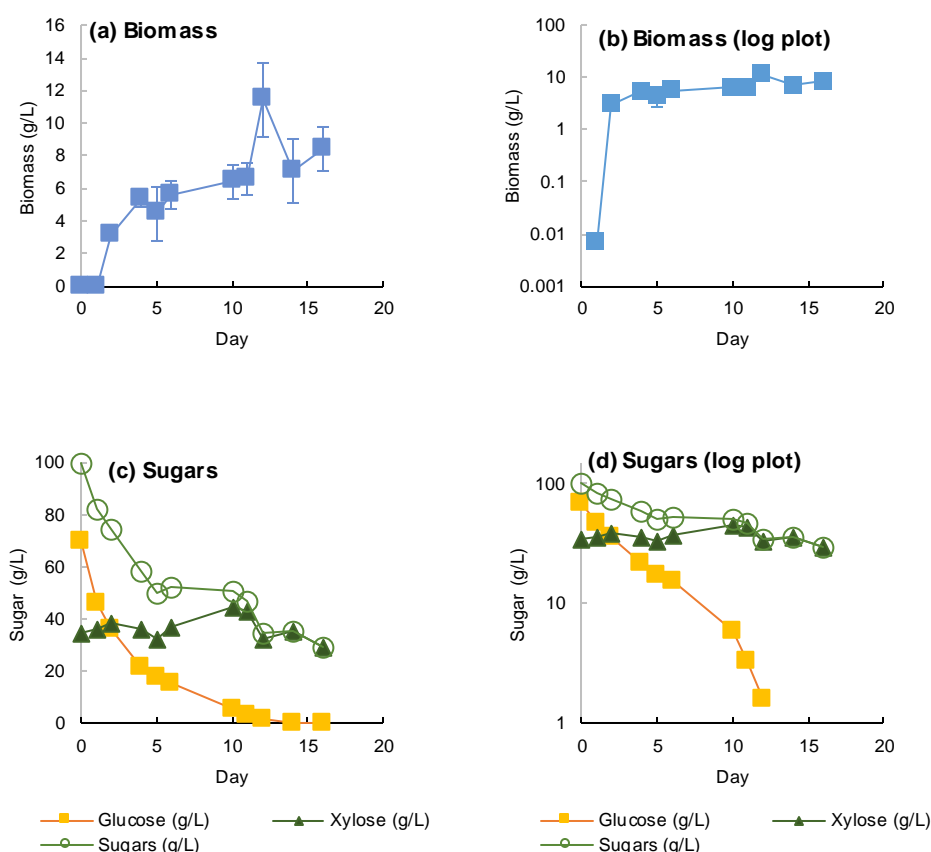


Figure 2.20 Growth and sugars utilisation from *M. pulcherrima* fermentation in modified SGJ media (glucose 70 g/L and xylose 30 g/L) at pH4, 20°C. (n=3, error bars represent one s.d.)

Growth of *M. pulcherrima* was found to be steady and reached the stationary growth phase in 4 days, possibly due to the abundance of glucose and nitrogen, shown in Figure 2.20 (a) and (b). However, from sugar analysis showed that the yeast utilised only glucose but not xylose. Glucose utilising rate, seen in Figure 2.20(d), was constant throughout the fermentation until day 10 where only a small amount of glucose was left. Xylose, on the other hand, was seen to be left unused even late in the fermentation when glucose was almost gone. This demonstrates that the glucose must be used entirely before the yeast with metabolise xylose. This could possibly result from the suppression from high glucose concentration at the start of the fermentation and affect the metabolism of xylose. In yeasts, xylose is transported in into cell and converted to xylitol and then converted to xylulose and xylulose-5P and enters the main carbon metabolism through

the pentose phosphate pathway (PPP). D-glucose suppression of this pathway is well known in *Candida* species relating to xylitol production [113-115].

Under these conditions, metabolic compounds such as arabitol and ethanol were produced as usual in the *M. pulcherrima* culture (Figure 2.21). Arabitol was produced up to 10 g/L and ethanol less than 2 g/L, though it was generated mostly from glucose as xylose was not utilised. 2PE production was not found in this xylose fermentation, possibly due to the nitrogen-rich condition which limits the carbon flux through shikimate pathway. As a result, phenylalanine, the essential precursor to 2PE production would not be created and, consequently, 2PE production was halted.

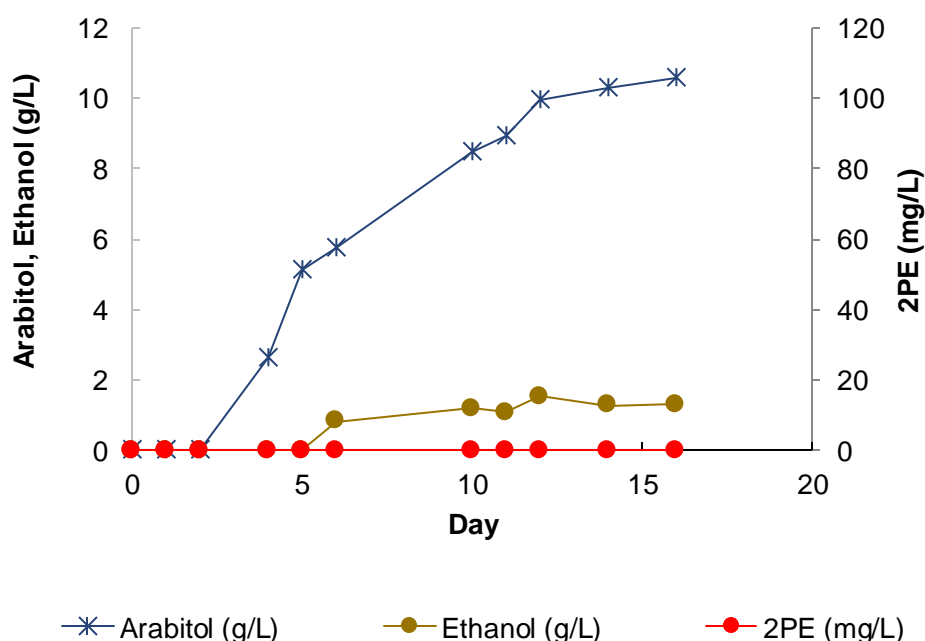


Figure 2.21 Products from *M. pulcherrima* fermentation in modified SGJ media (glucose 70 g/L and xylose 35 g/L)

Due to this negative results, it seems unlikely that 2PE production can be achieved from lignocellulosic feedstock easily. However, alternative waste feedstocks are available including glycerol, produced from biodiesel manufacture.

2.7.2 Glycerol

As biodiesel production is projected to be increase globally, the availability of glycerol as a carbon source for microbial industrial production platform is also increasing and upto 2 million tonnes could be available worldwide [116],[117]. This makes glycerol a good candidate as a substrate for high value chemicals from microbes.

Glycerol can be both substrate and the product from microbial fermentations. Several studies successfully used glycerol as carbon source for microbial fermentations as a new platform for metabolic engineering [118]. Glycerol is also a product from microbial fermentations, similarly to arabitol, because of osmotic stress response in the fermentation conditions such as in wine fermentation or in the saline environment. In the conditions of high osmotic pressure, the yeast creates the balancing chemicals such as glycerol and arabitol in the cells to balance the pressure with extracellular osmotic pressure. The osmoregulation and glycerol metabolism in *S. cerevisiae* yeast for example has been widely researched [119]. The recent advances in genetic models of yeast to osmotic shock and osmoregulation has also been well described [120, 121].

In wine fermentation, the sugar in wine can be up to 200 g/L which will create high osmotic pressure and resulted in only osmophilic yeasts which can control the osmotic pressure be active in the fermentation. This includes several yeasts found in common wine fermentation such as several yeasts in genera *Candida sp.*, *Hanseniaspora sp.*, *Issatchenkia sp.*, *Metschnikowia sp.*, *Pichia sp.* and *Saccharomyces sp.* [15]

Glycerol is synthesised from glucose via the Glucose-6P (G6P), Fructose-6P (F6P) and glyceraldehyde-3P which can either be converted to glycerol via DHAP and glycerol-3P or continued to TCA cycle. As these pathways are reversible, therefore they are also responsible for glycerol uptake as well. This will bring glycerol to the same metabolic pathway of glucose and theoretically should be able to substitute glucose for fermentation substrate.

M. pulcherrima was therefore cultured on glycerol and the biomass, 2PE, arabitol, and ethanol production assessed. The experiments were set up by growing *M. pulcherrima* in SGJ media with 25, 50, 75 and 100 g glycerol/L with glucose added up to make total substrate 100g/L in all media. The rest of the media was the same as SGJ media including tartaric acid buffer at pH4. The glycerol used was the crude waste from the transesterification of rapeseed oil (courtesy of the Croda Company). *M. pulcherrima* was cultured in 25 mL media in the 125 mL Erlenmeyer flasks in triplicate. The culture was shaken at 150 rpm in the incubator controlled temperature at 20°C. Glycerol and sugars were analysed by HPLC-RID with Biorad HPX-87H column.

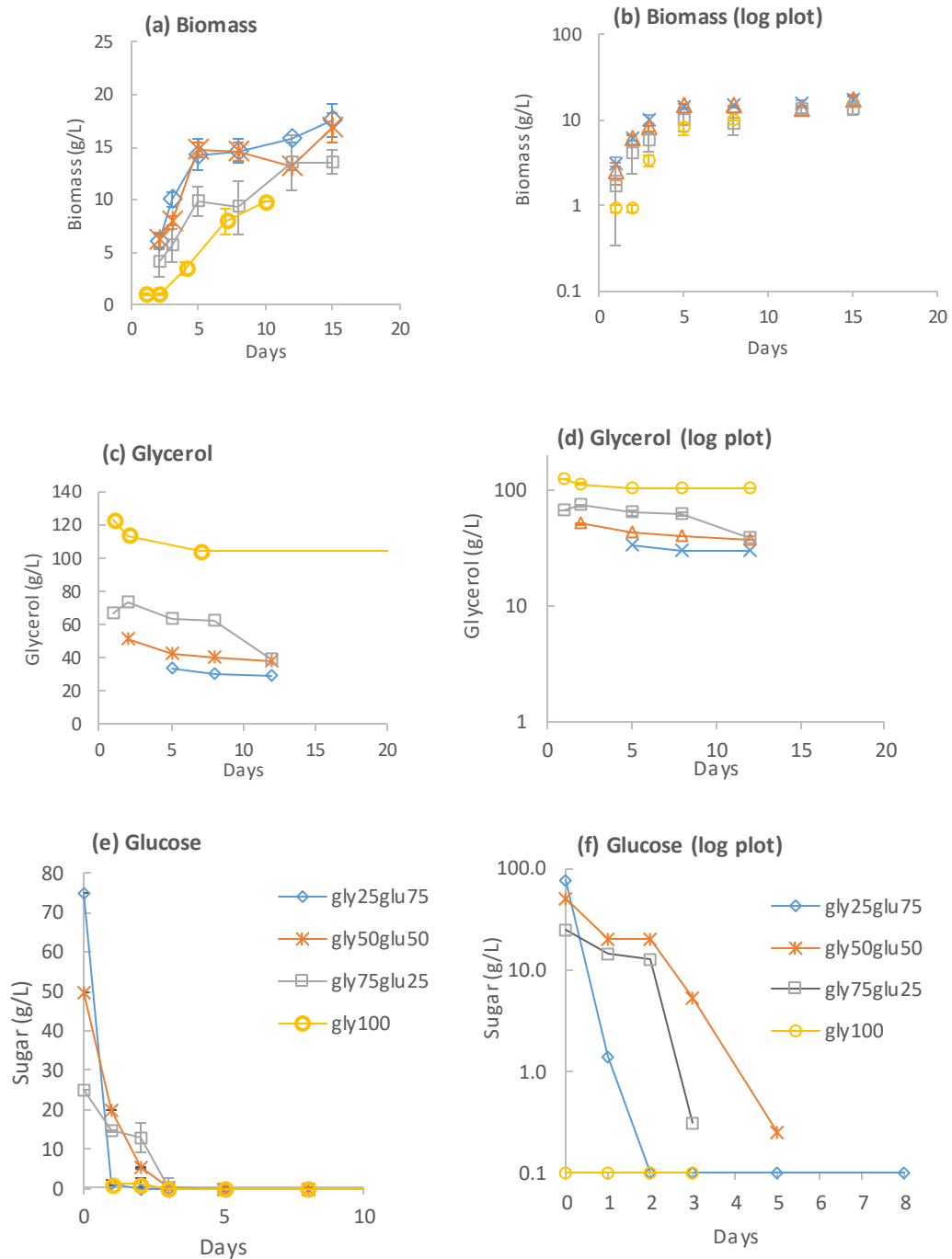


Figure 2.22 Biomass production, glycerol and glucose uptake in mixed glucose/glycerol SGJ media with different glycerol concentration pH4 at 20°C : (1) glycerol 25g/L+glucose 75 g/L, (2) glycerol 50 g/L + glucose 50 g/L, (3) glycerol 75 g/L glucose 25 g/L and (4) glycerol 100g/L. (n=3, error bars represent one s.d.)

In the SGJ media with glycerol as whole carbon source (100g/L), the growth of *M. pulcherrima* was lower compared to the glucose media (Figure 2.22). The maximum biomass achieved in the glycerol media is less than 10g/L after 10days, while the yeast grew between 12-15 g/L on the glucose media. It also grew far slower than SGJ media with glucose. This may because cellular glycerol uptake is slower than glucose. *M. pulcherrima* grew better in the mixture of glycerol and glucose. When glycerol is lower at

25 and 50 g glycerol/L (with glucose 75 and 50 g/L, respectively), the yeast growth was seemingly healthy. However, the difference was visible when glycerol was 75 g/L and over with less glucose. The growth at 75 g glycerol/L is slower and the maximum biomass is lower.

Though, the trace toxic chemicals from biodiesel production in glycerol may also have played a part in the growth inhibition of *M. pulcherrima* in this experiment as it usually contains some residual methanol and possibly NaOH. Seemingly, the effect from methanol should have been minimal, as no methanol concentration was detected in the broth by HPLC. The effect from the catalytic base as well should not affect the experiment because the media was previously pH adjusted. The growth phase lasted until day 5, not different from the glucose media. Therefore, for using glycerol as substrate, the reactor may need to be designed with a slower rate of growth in mind.

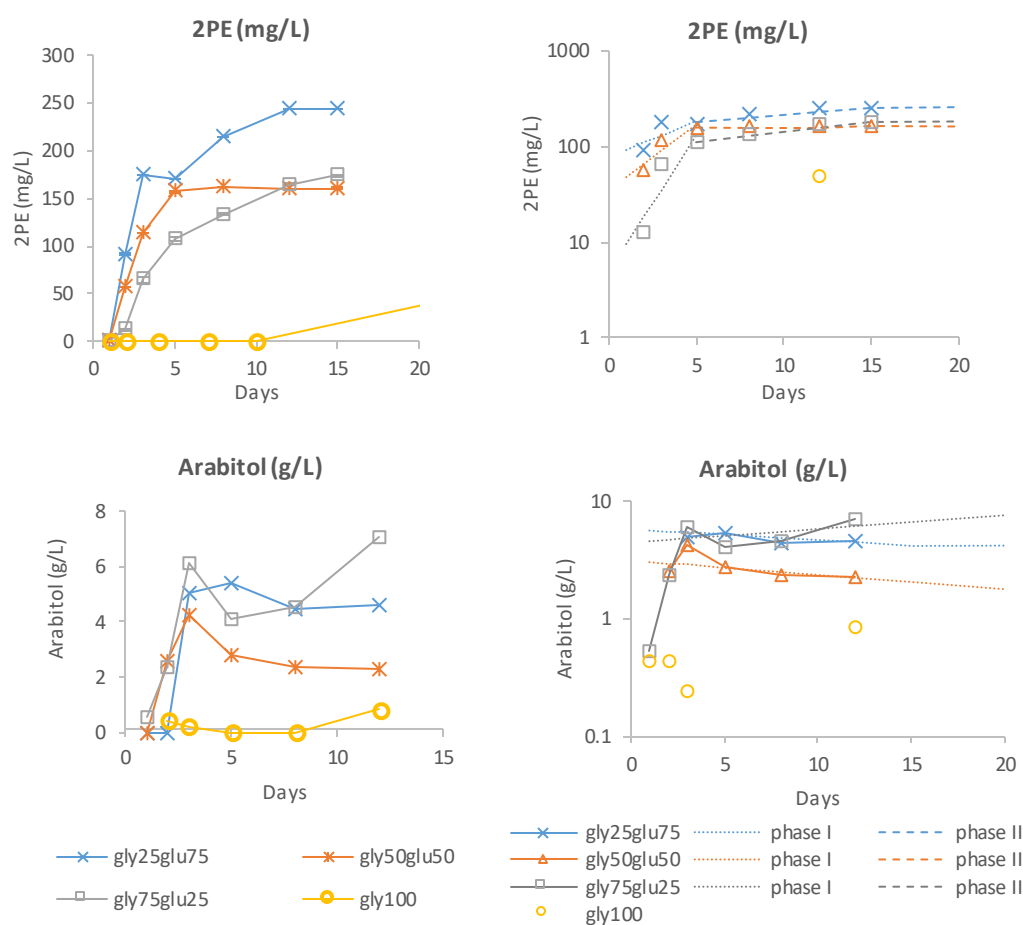


Figure 2.23 2PE and arabitol production from SGJ media with different glycerol concentration pH4 at 20°C : (1) glycerol 25g/L+glucose 75 g/L (2)glycerol 50 g/L + glucose 50 g/L (3) glycerol 75 g/L glucose 25 g/L (4) glycerol 100g/L

Similarly, 2PE and arabitol production by *M. pulcherrima* was decreased when increasing glycerol was used (Figure 2.23). Hardly any 2PE was observed when only glycerol was used, and this was after 20 days. However, with the high biomass created, it suggested the presence of nitrogen source in waste glycerol, which would promote the growth but limit the production of 2PE, due to de novo 2PE production through shikimate pathway needs to be triggered by the low nitrogen content to stimulate the synthesis of L-phenylalanine. It could indicate that nitrogen may present in the waste glycerol, presumably from used vegetable oil resulted from meat cooking which could leave nitrogen compound in the used vegetable oil and subsequently in waste glycerol. Therefore, when waste glycerol was more, 2PE was produced less as it increased the amount of nitrogen in the medium. However, high biomass production in *M. pulcherrima* may suggest waste glycerol use as alternative substrate could still be possible, further investigate for 2PE production may be needed, though.

2.8 Conclusion

M. pulcherrima has recently been reported as a lipid producer. In this study it was demonstrated that, in addition, the yeast could produce 2-phenylethanol, the high value aroma compound used in fragrances, as well as arabitol, the pentose sugar that could be the important building block chemicals for bio-refinery.

The fermentation was optimised to produce 2PE and arabitol. It was found that the best conditions for 2PE production were at pH4, 20°C with high glucose loading (100 g/L). Nitrogen ratio was also found to be an important parameter for 2PE production and nitrogen content 0.67 g/L (C/N ratio is approximately 0.2 in the media with 100 g/L glucose) was found to be the optimal under these conditions. Arabitol on the other hand seems to be produced in higher stressed conditions. The maximum production of arabitol was found to be at pH3, 20°C and 100 g/L glucose which yielded over 10 g/L arabitol. Higher temperature could result in a higher arabitol production, had it not stopped the fermentation activity. Variation of productivity among the different strains was observed as well, therefore, strain screening is one of the important step in the fermentation. The optimal condition of 2PE and arabitol production from *M.pulcherrima* is summarised in table 2.6

Table 2.1 optimal condition for 2PE and arabitol production from *M.pulcherrima*

	2PE	Arabitol	Biomass
pH	4	3	5
Temperature (°C)	20	28	20
N/C ratio (g/g)	0.2	0.2	1
Glucose (g/L)	100	100	100

The highest yield of 2PE was found to be 1.0 g/L, two magnitudes above the production by *M. pulcherrima* in wine fermentations, and higher than any other non-GMO in the literature. Ethanol and 2PE were investigated for their inhibitory effect towards the yeast growth. It was revealed that the 2PE tolerance of *M. pulcherrima* is 1.8 g/L but decrease to 1.2 g/L with the presence of ethanol 4% or higher. When not in the presence of 2PE, *M. pulcherrima* can tolerate ethanol up to 12% or higher. The concentration of both compounds in the *de novo* synthesis fermentation are far lower than this, and it seems that the fermentation is not limited by these compounds. While culturing on glucose gave excellent yields, both xylose and glycerol were found to be less effective.

It is therefore clear that *M. pulcherrima*, cultured on glucose, has the potential as a microbial producer of 2PE. In the next chapter the suitability of this fermentation to a larger scale was investigated with the scale up of the yeast to stirred tank 2-L bioreactors.

2.9 References

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Chapter 3

De novo 2-Phenylethanol Production
from *Metschnikowia pulcherrima* in
Semi-Continuous Culture and
Scale up of 2L Stirred Tank Bioreactor

3.1 Introduction

In the previous chapter, *M. pulcherrima* was shown to be able to produce 2PE directly from glucose in high titres. However, to be able to produce 2PE on the industrial scale the process must be demonstrated to be scalable in controlled 2-L stirred bioreactors. Over the course of this chapter the 2PE production from *M. pulcherrima* was assessed in a 2L bioreactor and in semi-continuous and continuous modes.

Fermentation in controlled bioreactors is different from lab-scale shake flasks in many ways such as oxygen transfer, heat transfer on temperature control, mixing and agitation in the fermentation. Also some characteristics in larger scale fermentations such as foaming, the cells sensitivity to shear force and the oxygen requirement are problematic on the larger scale and are not realised in flasks [122].

For these reasons it is common for results to differ substantially between the flasks and bioreactors [123]. In the bioreactors parameter control is achieved via an automatic controller, offering far greater control that cannot be achieved in shake flask experiments. Data logging during fermentation on the larger scale also reveals more data on how the fermentation proceeds during the course of the experiment.

One key parameter that can be controlled with the 2L bioreactors, which cannot be in shaking flasks, is the aeration. Along with carbon source and nitrogen source, the oxygen is one of the important factors that can control the growth and the yeast metabolism during fermentation [124]. Most of the yeasts are facultative which can survive in aerobic and anaerobic conditions as they possess both aerobic and anaerobic metabolic pathways [125, 126]. However, the anaerobic pathway generates less energy per mole as the process lacks electron acceptors such as oxygen and the ATP was generally generated only in the glycolysis process and ethanol fermentation. In contrast, under aerobic conditions, with oxygen as electron acceptor, substrate would be transferred completely through the tricarboxylic cycle yielding CO₂ and 34 ATPs per mole glucose [127]. Therefore, the growth and metabolism of the yeast are highly affected by the aeration.

The production of 2PE was therefore undertaken on an Electrolab 2L automated bioreactor where the temperature, pH, agitation and aeration were all controlled. Temperature was controlled by hot jacket and cooling coil with running cool water. pH was controlled by the automated addition of 1M HCl and 1M NaOH, regulated by the controller and the pH probe. Agitation was provided by a double vertical 6-blade turbine which could be set to a specific rpm. The aeration flow rate was controlled through a rotameter and regulated by the controller. The level of dissolved oxygen was designed to be kept constant by either regulating on-off air inlet according to the dissolved oxygen,

or controlling the speed of agitation. However, it was not always practical as during the high oxygen consumption such as growth phase, the air supply failed to match the high need of oxygen during the time and, consequently, the dissolved oxygen can be lower than desired (Figure 3.1). The aeration in the experiment was then set up by control of the air inlet flow rate and the dissolved oxygen was set as one of the dependent variables.

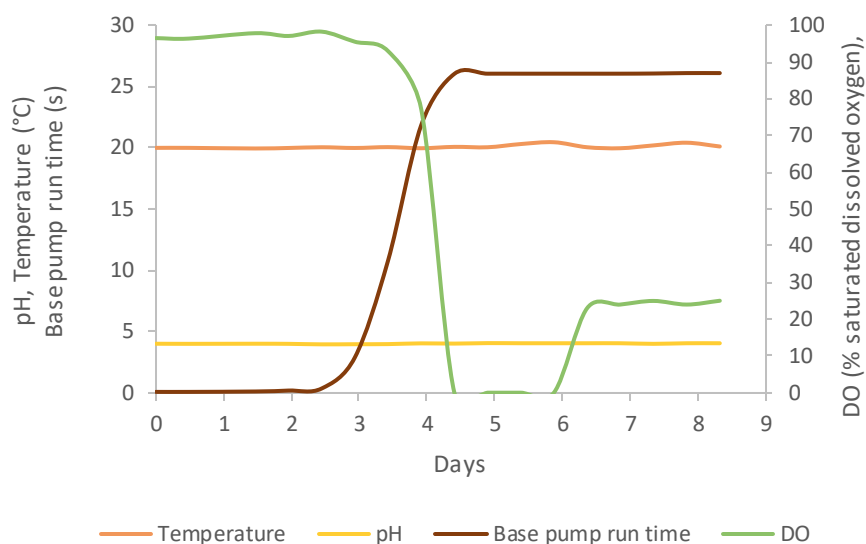


Figure 3.1 Logged temperature, pH, dissolved oxygen (DO) and base pump run time in *M. pulcherrima* fermentation in SGJ media the 2L controlled bioreactor at pH4, 20°C, agitation speed 150 rpm and dissolved oxygen at 25% saturated dissolved oxygen. pH was controlled by automatic addition of HCl 1M and NaOH 1M

3.2 Effect of aeration on the *de novo* 2PE production from *M. pulcherrima*

Initially the effect of aeration on the production of 2PE from *M. pulcherrima* in 2L fermenter was investigated (Figure 3.2). The study of aeration rate in *de novo* 2PE production from *M. pulcherrima* was carried on with fixing the air inlet flow rate to the bioreactor. The aeration was fixed constantly during the experiment for 0.5, 1 and 2 L/min, with the working volume 2L in the bioreactor. The maximum aeration rate at 2L/min represented 1 working volume/min in the bioreactor. The conditions of fermentation were taken from the optimised shake flask experiments from the last chapter and were set at pH 4, 20°C with an agitation speed of 180 rpm. The bioreactor jar and lid were autoclaved prior to fermentation.

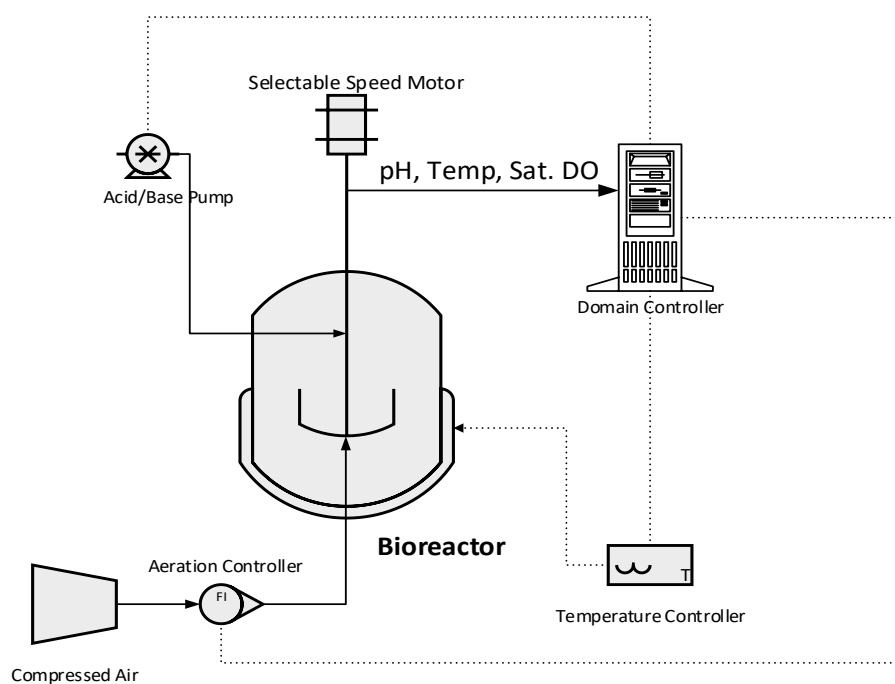


Figure 3.2 Controlled Batch Bioreactor

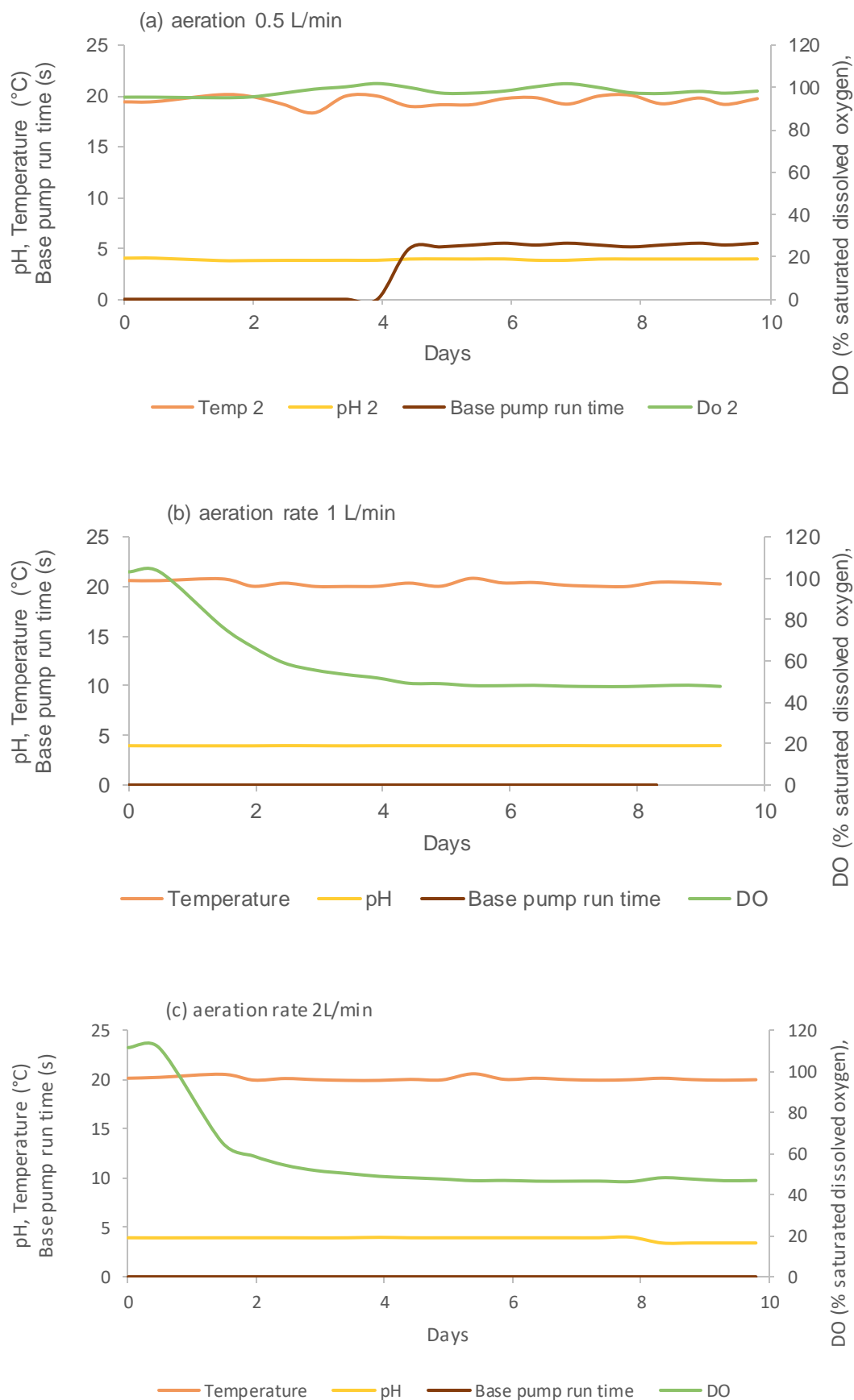


Figure 3.3 Fermentation profiles including temperature, pH, DO and base pump run time in the *M. pulcherrima* fermentation in 2L bioreactor at the aeration rate 0.5, 1 and 2 L/min. The condition of the bioreactor was kept at 20°C and pH4

The fermentation profile of the conditions in the bioreactor during the different aeration rates are shown in Figure 3.3. The conditions in the bioreactor such as pH and temperature were well controlled and remained at pH 4 and 20°C throughout the experiment. The dissolved oxygen (DO) dropped substantially during the growth phase of the yeast during the experiments with 1 and 2 L/min in the first few days of the fermentation, and never went above 50% after this point. The DO in the fermenter with 0.5 L/min remained approximately at 100%, possibly due to the lower biomass than the other experiments.

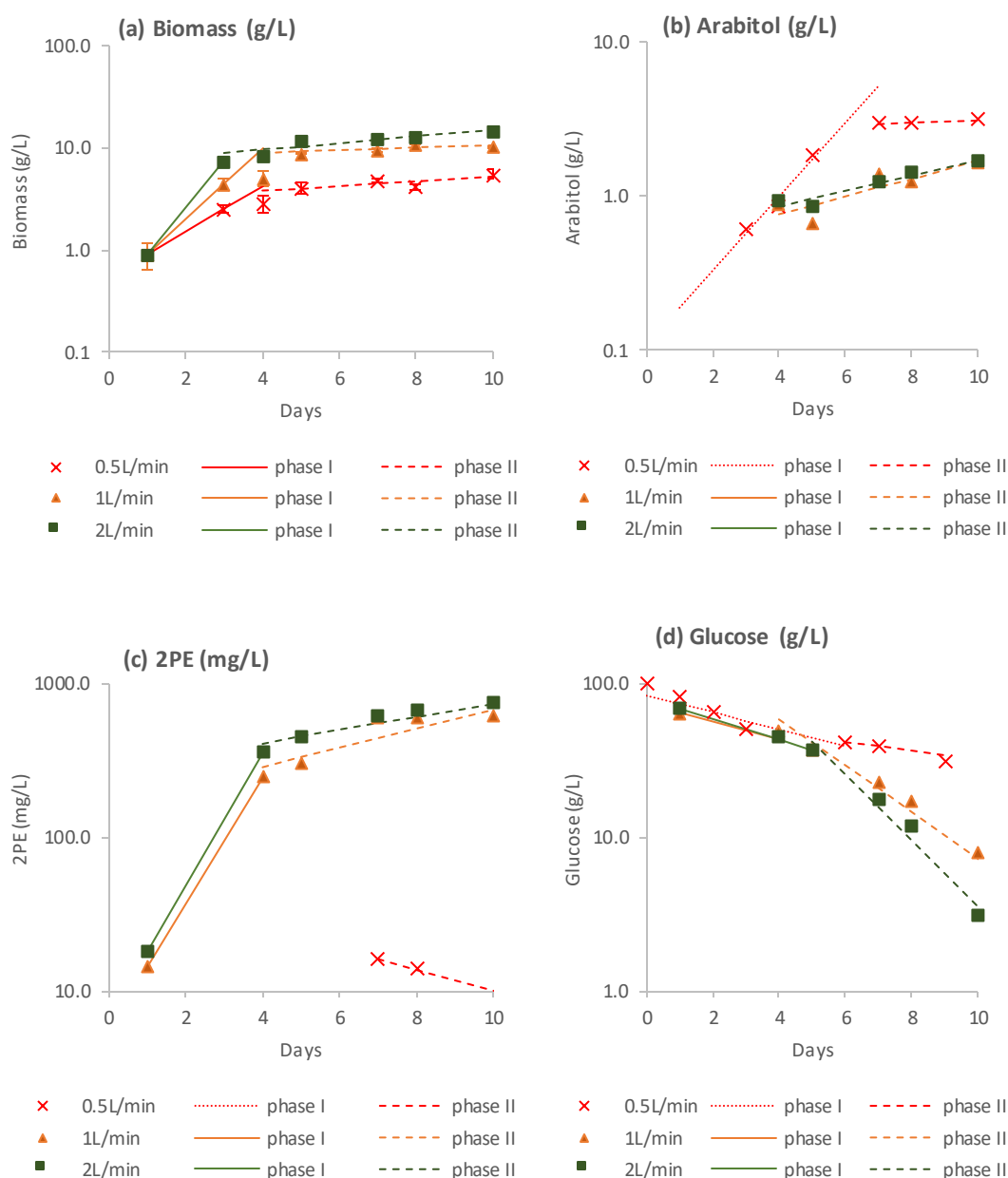


Figure 3.4 Effect of aeration to growth and metabolic products in *M. pulcherrima* culture in SGJ media at pH4, 20°C in 2L bioreactor

The biomass and 2PE productivity achieved were similar in this bioreactor set up to the shake flasks, with up to 14 g/L of yeast biomass, 700 mg/L of 2PE and 3.5 g/L of arabinol produced depending on the conditions (Figure 3.4). At all aeration rates examined, the yeast was able to produce both 2PE and arabinol. However, the aeration rate did affect the activity with changes in both the growth kinetics and the fermentation products.

For example, the aeration at 0.5L/min seemed to be too low for the fermentation in general and lowered the growth rate substantially, generating approximately 5 g/L of yeast biomass. This was only half of the biomass the yeast normally achieved in the stationary phase. The low aeration rate also adversely affected 2PE production, reducing the final 2PE titre from over 500 mg/L observed in the shake flask experiments to 20 mg/L. This seems to be caused by the limited aeration rate forced the yeast to metabolise glucose via the anaerobic pathway which generate 2 ATPs/mol glucose during the glycolysis, compared to up to 36 ATPs/mol glucose in the aerobic metabolism where oxygen is presented as an electron acceptor. In the anaerobic phase, the product is ethanol which was observed throughout the fermentation.

The limited energy due to the anaerobic phase led to the reduced formation of amino acids and building new cells. This is different from limitation of nitrogen which can have the negative effect on the cell building as it was seen the chapter 2, as the amino acids and the new cell production were inhibited by limitation of nitrogen source which is necessary to synthesise amino acids. But it did lead to the same result which is limitation of growth and desired metabolic compounds production. Similar to the work presented in the previous chapter, low 2PE production and high ethanol production also corresponded with higher arabinol production.

The higher aeration rate of 1 L/min (half the working bioreactor volume per min), was more suitable for the yeast to grow healthily and for the general production of metabolites. The yeast biomass achieved was 10 g/L over the fermentation, double that with the lower air rate. 2PE production was also significantly increased to 600 mg/L by the end of the fermentation which is in the range of the production found in the lab-scale shake flasks.

On increasing the aeration further, to 2 L/min, the yeast biomass was increased to 14g/L, approximately 3x that produced at low aeration. In addition, 700 mg/L of 2PE was recovered, similar to the titres produced on the batch shake flask experiments presented in chapter 2. The amount of produced 2PE by *M. pulcherrima* in the bioreactor is satisfactory compared to other *de novo* 2PE production from other organisms. And while the cost of aeration can be a significant cost in a fermentation it seems clear that this level of aeration is vital for the culture and the 2PE production metabolic pathways.

In batches with higher aeration, the arabitol production is noticeably reduced (Figure 3.4b). The higher arabitol production at low air rate flows could be because arabitol production is a response to environmental stress such as limited oxygen in *M. pulcherrima*. For example, Kayingo et al. demonstrated that the arabitol-producing yeast *Candida albicans* (which is taxonomically close to *M. pulcherrima*) produced both glycerol and arabitol as a stress response under adverse environmental conditions. In this yeast glycerol is produced under osmotic stress conditions and arabitol under oxidative and temperature stress [128].

The yeast biomass and compounds produced occurred in two phases in all experiments, the log plots are shown in Figure 3.4. The rate of biomass accumulation in the growth phase was lowest for the aeration rate 0.5 L/min and 1 L/min, though both experiments the yeast entered the stationary phase only on day 4. The rate was a little faster for the culture aerated at 2 L/min which entered the stationary phase by day 3. This has shown that the higher aeration rate helped the yeast grow better.

2PE was produced predominantly in the growth phase (Figure 3.4c), with the rate being higher for the higher aeration, similar to batch experiment in shake flasks where high glucose with a nitrogen deficiency triggered the shikimate pathway to synthesise phenylalanine which was subsequently converted to 2PE via the Ehrlich pathway. On the other hand, arabitol seemed to be produced throughout the fermentation as a metabolic compound regularly from osmotolerant yeasts. However, the effect of aeration on the arabitol production is less well understood [95, 129, 130].

On the substrate uptake, glucose seemed to be consumed differently between the growth phase (day 0-4) and stationary phase (day 4-end) as shown in Figure 3.4 (d). Glucose in all 3 aeration rates was consumed at the same rate in the growth phase while, in the stationary phase, the uptake rates differ substantially (Figure 3.5), where glucose seemed to be consumed at a much higher rate in the higher aeration.

It can be hypothesised that the glucose uptake in *M. pulcherrima* are regulated by the extracellular glucose concentration which is supported by [131], regardless of the aeration rate, therefore glucose was consumed by the yeast in the similar rates in the initial stage. In the higher aeration rate, glucose is metabolised through tricarboxylic cycle in a faster rate due to availability of aeration, therefore, it could induce cellular glucose transport at the higher rate [132-134].

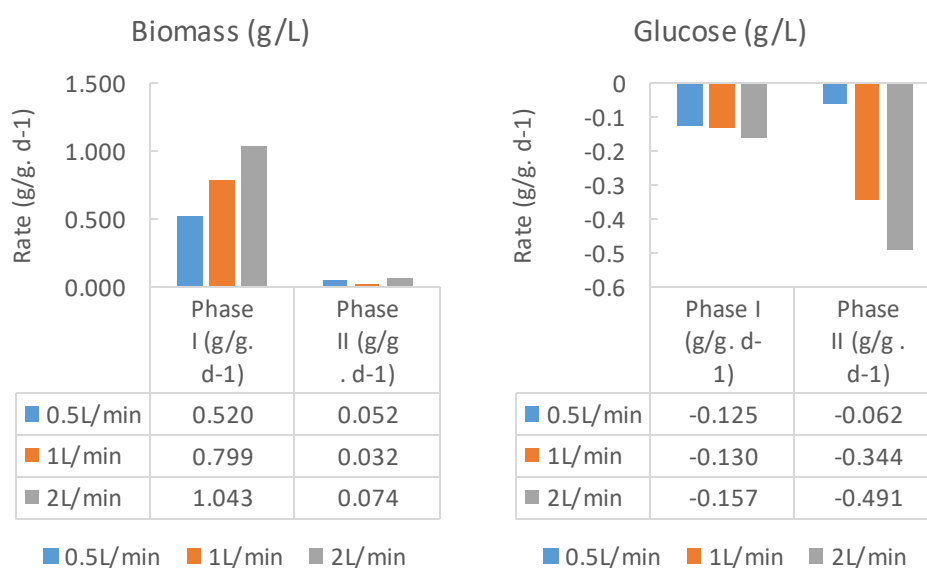


Figure 3.5 Growth rate and glucose utilisation rate of *M. pulcherrima* in different aeration regimes in 2L controlled bioreactor at 20°C, pH4 (phase I and II are growth phase and stationary phase, respectively)

The end products during different aeration rates are summarised in Figure 3.6. The figure shows that the higher aeration rate of 2 L/min (one working volume of bioreactor per minute) produced the most biomass and 2PE. Though this is a positive result, it must be balanced with the extra cost of providing aeration to the reactor.

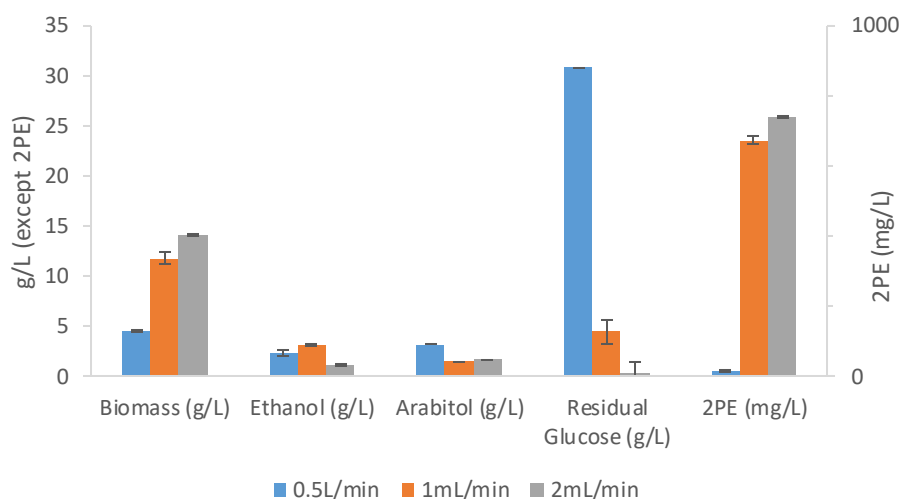


Figure 3.6 Products and residual glucose at the end of *M. pulcherrima* fermentation in SGJ media in 2L controlled bioreactor at pH4, 20 °C, error bars represent sampling replicates from the fermenter (n=3)

3.3 Semi-continuous *de novo* 2PE production from *M. pulcherrima* in shaking flasks

In chapter 2, *M. pulcherrima* was shown to be able to produce 2PE in a batch mode in shake flasks and, with high aeration, is scalable to at least the 2L bioreactor scale. However, the compounds, especially 2PE, were produced mostly during the growth phase. This is because it requires two connected metabolic pathways to create 2PE from glucose. Firstly, the Shikimate pathway is used to convert glucose into L-phenylalanine and, secondly, L-phenylalanine needs to be converted to 2-phenylethanol via the Ehrlich pathway [30]. The Shikimate pathway is the pathway found in bacteria, yeast and plants that the organisms use to create aromatic compounds which are mostly aromatic amino acids such as phenylalanine, tyrosine and tryptophan in bacteria and yeasts. In plants, it was one of the most important pathway and used extensively to create aromatic compounds needed in plants such as quinones, folates, alkaloids, flavonoids and lignin. [29] The shikimate pathway is found to be regulated by nitrogen deficiency [37], therefore, it is likely to be the bottleneck of *de novo* 2PE production from glucose, rather than the Ehrlich pathway which is not limited by particular conditions [135]. Such nitrogen-limited conditions occur in the exponential growth phase, usually at the end of growth phase, where amino acids are extensively synthesised and nitrogen deficiency stimulates the carbon flux through the shikimate pathway, resulting in phenylalanine synthesis in high titres. As a result, 2PE which is converted from phenylalanine is synthesised mainly in the growth period [30, 136].

However, on the batch mode there is a significant lag phase, where the yeast is acclimatising to the conditions and media. This is unavoidable under these operating conditions though can be potentially removed by producing the 2PE in a semi-continuous mode instead. This would also benefit from being able to produce 2PE in smaller reactor volumes with less down time of the bioreactor.

A semi-continuous fermentation is when the broth in the fermentation is partially removed from the fermenter periodically following the addition of fresh new media. The yeast can then use the nutrients in the fresh media to generate new biomass replacing what has previously been removed in a rapid timeframe with no lag time [137]. It is similar to using a continuous reactor on dilution ratio or mean cell residence time (MCRT) which can be controlled by how much the replaced and removed volume is applied to the fermentation, similar to the feeding flow rate of continuous process. [138]

Semi-continuous fermentation presents some advantages over batch and continuous process. It usually contains higher biomass than in batch fermentation and, consequently, higher product formation rate and a lower fermentation time. It also is less

prone to flush out than continuous process which can be controlled by the feeding and the removal volume of the fermentation [139]

The initial semi-continuous experiment was conducted using a 50mL tube with working volume of 15 mL, with the similar ration media to air as in the previous chapter. The tubes were sterilised and capped with a sterile sponge for effective gas transfer. Two types of fermentation were examined in the first the aliquot of media removed contained biomass which was then discarded. This was then compared to the yeast fermentation where the biomass was not discarded but retained in the vessel. This was achieved by centrifugation of the removed supernatant and the removal of the biomass pellet. The culture tubes were incubated at 20°C with the SGJ media pH 4 with tartaric acid 7 g/L as a pH buffer and internal standard. The tube was placed in the 2.5-inch cup and shaken at 150 rpm, providing higher shaking and air transfer. The supernatant was removed and replaced according to the calculation of dilution ratio 1/3, 1/6, 1/9 and 1/12 or hydraulic retention time (HRT) 3, 6, 9 and 12 days.

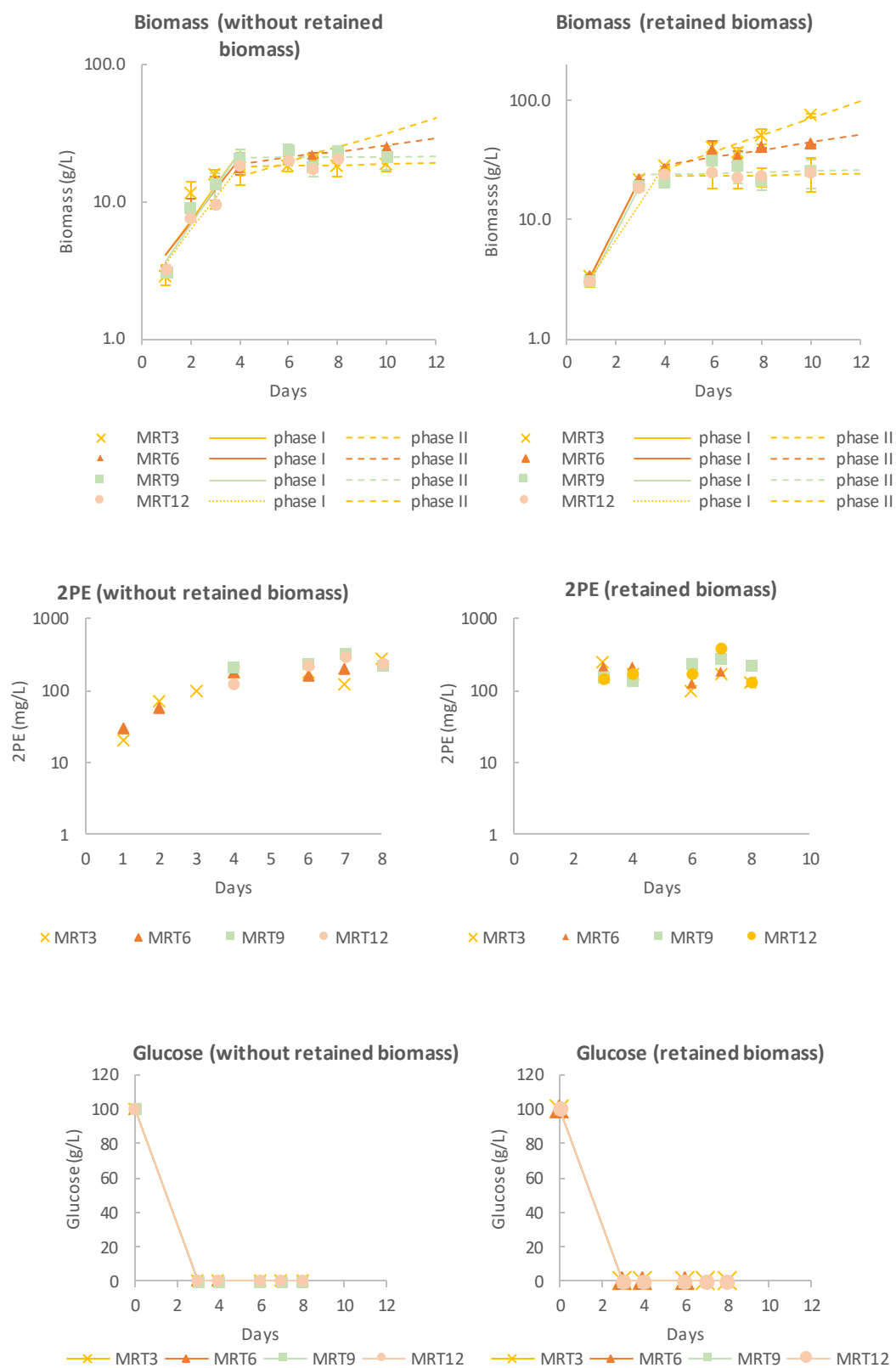


Figure 3.7 Growth, 2PE and sugar utilisation in semi-continuous shake flask culture with *M. pulcherrima* with hydraulic retention time (HRT) 3, 6, 9, 12 days (dilution rate 1/3, 1/6, 1/9 and 1/12 day⁻¹, respectively). Semi-continuous process started on day 3. (n=3, error bars represent one s.d.).

The biomass, 2PE and glucose utilising from *M. pulcherrima* in semi-continuous culture is shown in Figure 3.7. The biomass in different dilution ration in the culture without retained biomass is similar in the growth period but the difference is visible in the phase II in the stationary period. The culture with higher dilution ratio saw the higher biomass production, presumably as there was more fresh media available. Therefore, a dilution ration of $1/3 \text{ day}^{-1}$ (the culture was taken out $1/3$ of the working volume each day and added with fresh media at the same volume), had the higher growth in the stationary period. Less biomass was achieved in $1/6$, $1/9$ and $1/12 \text{ day}^{-1}$, respectively.

Naturally, the biomass in the retained system is higher than in the culture without retained biomass process, which in this experiment they were centrifuged before removal and additional fresh media added. With the retained biomass system, the highest biomass achieved was close to 75 g/L after 10 days which is approximately 6-12 times the usual biomass concentration achieved in any other cultures. The retained system can possibly be done in the larger scale by letting the culture settle before the broth replacement. Though this higher biomass loading would likely reduce the oxygen transfer and mixing degree of the culture.

The yeast in both systems seemed to produce 2PE to a similar amount, approximately $170\text{-}250 \text{ mg/L}$ and there was no significant difference between dilution rations. The unusually low 2PE concentration may have resulted from the higher aeration rate in the shaking 50 mL tube. It also resulted in the higher-than-usual biomass production in the non-retained biomass culture as the higher aeration supplied more oxygen in the metabolism which yields more ATPs for new cell generation but less 2PE production. The higher rate metabolism was also seen in the glucose uptake rate in both systems where the glucose was uptaken so rapidly that none was left in the culture by the end of the day. This high glucose uptake rate has not been seen in other experiments. Ethanol and arabitol were barely produced in any of the experiments and, therefore, were not shown.

Glucose was uptaken so quickly in this experiment, it had not been detectable since the semi-continuous process started (day 3) both in the retained biomass and without retained biomass. This is possibly due to the higher than usual biomass building up in the culture, especially in the retained biomass which the cultures were centrifuged before the supernatant was taken out, resulted in the competitive condition for substrate in the culture. When new media was added in, therefore, sugar was adsorbed rapidly. In further investigation of glucose uptake rate in this condition, it was found that glucose was used up in the first two hours in the retained biomass semi-continuous culture. (data not shown)

3.4 Continuous 2PE production in 2L bioreactor from *M. pulcherrima*

M. pulcherrima is capable of producing 2PE in batch shake flasks, in 2L bioreactors and can be grown in extremely thick yeast cultures in a semi continuous mode. 2PE production is mainly observed in the growth phase where the rate of 2PE production is high, though is barely observed in the stationary phase. In the growth period, the yeast growth was triggered by abundant glucose and the limited nitrogen source has shown to increase the production of aromatic compounds, due to the phenylalanine ammonia-lyase (PAL) activities governing shikimate pathway presumably as a response to nitrogen deficiency. [140, 141] Most of the *M. pulcherrima* in SGJ media have the growth span of 3-5 days.

Batch operation in the bioreactor provides certain advantages such as simple control over biomass. However, the batch process presents some drawbacks such as unavoidable lag time and accumulation of inhibitors or toxic compounds during the fermentation time. Other bioreactor regimes are also considered if they can provide advantages over batch experiments such as a shorter fermentation time, higher production rate, smaller bioreactor volume or less aeration. To examine the effectiveness of this approach, and the likelihood of the process being scalable, the continuous production was undertaken using a 2L bioreactor (Figure 3.8).

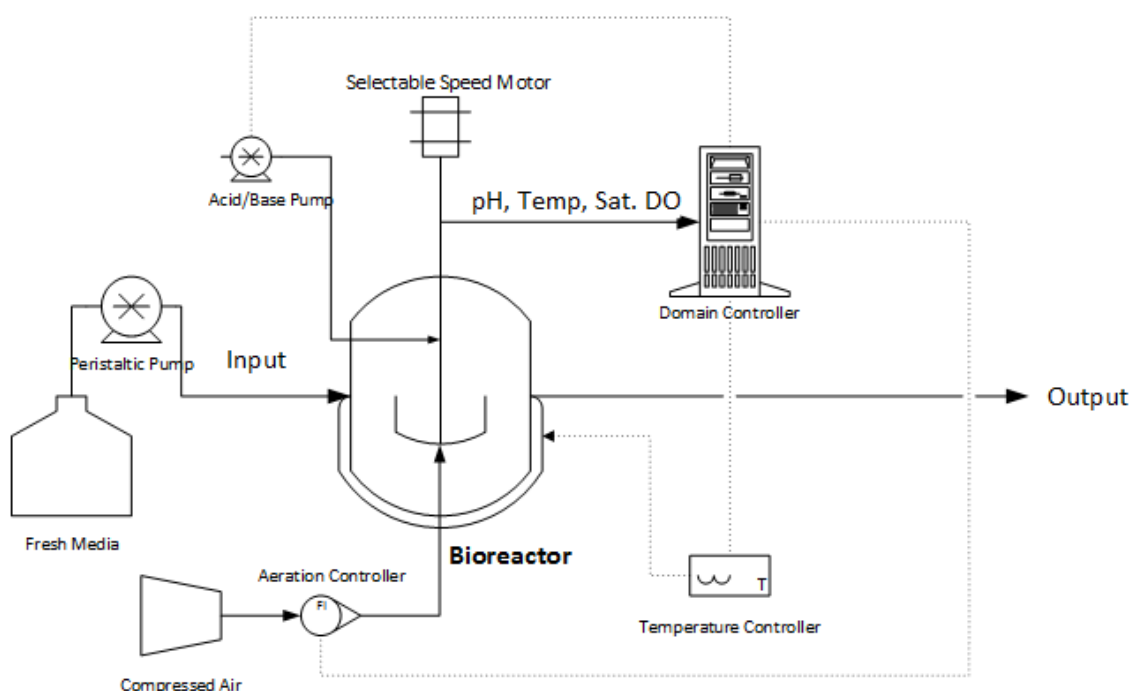


Figure 3.8 Continuous bioreactor for 2PE production from *M. pulcherrima*. The condition was automatically controlled at 20°C by cooling coil and heating blanket and pH4 by adding HCl 1M and NaOH 1M

The fermentation was conducted in the same 2L bioreactor as prior experiments detailed in section 3.2. The experiment was carried out by starting in batch mode for 7 days and continued in continuous mode from day 8. During the continuous period, sterile fresh SGJ media was fed into the bioreactor by the peristaltic pump. The flow rate was controlled by the on/off switch operated in cycle (10 minutes/cycle), regulated by the main controller. The condition was controlled at 20°C automatically and pH 4 was maintained by adding HCl 1M and NaOH 1M, regulated by the main controller as well. The continuous phase was carried on with dilution rate 8 hours or $1/3 \text{ d}^{-1}$ (hydraulic retention time, HRT 3 days) as it should provide the mean biomass retention time 3 days in the chemostat approximately the span of growth period in batch fermentation.

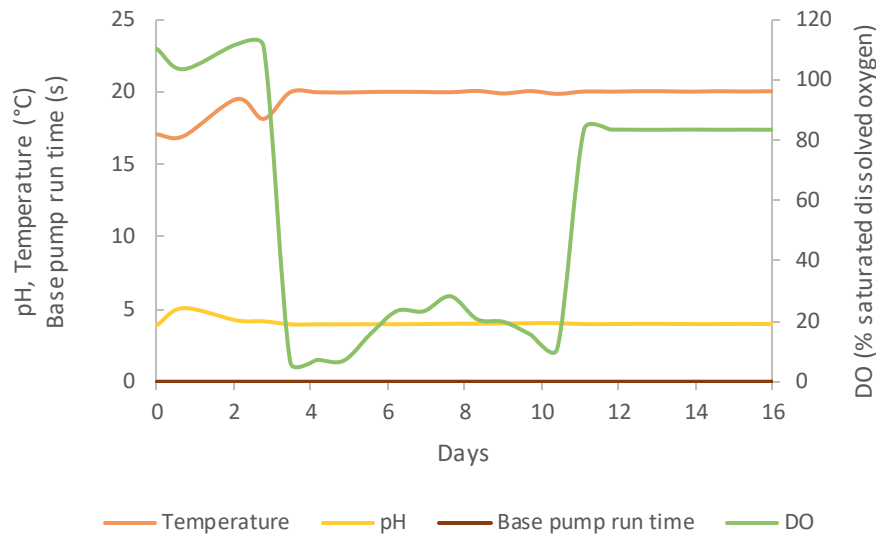


Figure 3.9 Temperature, pH and DO profiles in continuous *M. pulcherrima* fermentation with dilution ratio $1/3 \text{ d}^{-1}$ (HRT 3 days) at 20°C , pH4. The bioreactor was run in batch mode from start to day 7 and continuous mode since day 8.

The environmental conditions including temperature, pH and DO of *M. pulcherrima* during the continuous fermentation is shown in Figure 3.9. After day 3 the DO dropped substantially demonstrating that the yeast had entered the exponential phase.

M. pulcherrima grew very well in SGJ media as is usual in the batch mode, with a maximum biomass close to 20 g/L while producing up to 500 mg/L 2PE by day 8 (Figure 3.10). On day 8, the fresh sterile SGJ media was fed to the bioreactor and the fermentation started to be run in the continuous mode, with the overflow pumped out from the bioreactor to keep the volume constant. The performance of the reactor in continuous mode is shown in the yellow phase in the figure.

The yeast biomass decreased by approximately a third in the first day of continuous running suggesting the biomass was washed out with the overflow output. The reduced ratio biomass tallies with the dilution ratio of $1/3 \text{ d}^{-1}$. The new yeast cells seemed not be able to reproduce fast enough to replace the lost cells in the first day of the continuous mode. However, the biomass recovered after this point and increased up to 12 g/L by day 11. After this point the culture appeared to crash, with only 1 g/L of yeast present in the bioreactor after day 15.

2PE level in the bioreactor was also affected by the dilution at first but in the first 24 hours of continuous running the yeast is producing 2PE and by day nine almost 600 mg L^{-1} was present. As the yeast biomass started to rise up, 2PE concentration rose as well. The risen 2PE concentration was higher than during the batch period suggesting the new media added in the continuous process made the yeast produced new cells and by the same time the 2PE production via shikimate pathway was also triggered. The continuous

regime also means that signalling compounds and other inhibitors accumulate less in the bioreactor resulting in higher product formation. As a result, 2PE was more produced and the concentration was increased to over 1600 mg/L.

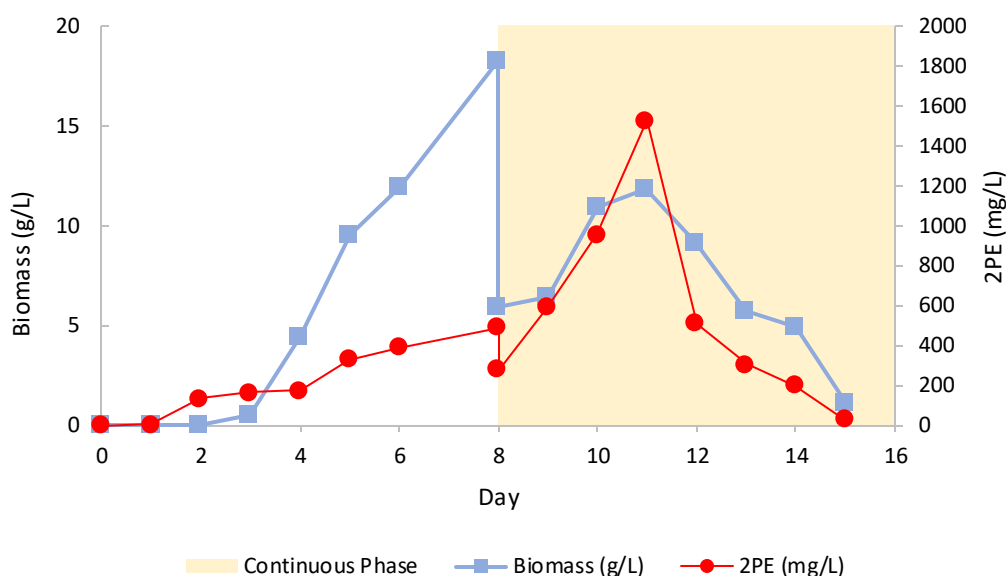


Figure 3.10 Growth and 2PE production from *M. pulcherrima* in continuous bioreactor with dilution ratio $1/3\text{ d}^{-1}$ (HRT 3 days) at 20°C , pH4. The bioreactor was run in batch mode from start to day 7 and continuous mode since day 8.

After 3 days in the continuous phase, the accumulated 2PE is just too high, with the limiting threshold of 1500 mg/L being reached. This is presumably the cause of the yeast death, explaining the rapid decrease of biomass concentration.

The continuous flow bioreactor was shown to increase 2PE production substantially on the batch process, and the yeast initially responded well to this mode of operation and the new media in the first few days. In the dilution ratio $1/3$ of working volume per day, that made the mean yeast cell resident time in the bioreactor 3 days and was shown to be ideal for 2PE production. However, when the dilution rate was high and the yeast was operated at young mean age, it increased the 2PE too much to the inhibitory level to the yeast and caused the flush out since day 11. Therefore, a lower dilution rate would be needed to retain a longer biomass retention time in the bioreactor, and create a more stable 2PE platform.

Further study on continuous bioreactor with the longer hydraulic retention time was conducted whether to improve the performance of the continuous fermentation with HRT 6 days. The growth and 2PE production of *M. pulcherrima* in continuous mode with dilution rate $1/6\text{ d}^{-1}$. It was started in batch mode over the first 8 days, similar to the continuous mode at a dilution of $1/3\text{ d}^{-1}$, and after that in continuous mode since day 8. The profile of *M. pulcherrima* fermentation during the continuous regime with HRT 6

days, given in Figure 3.11, shows that while pH and temperature at 4 and 20°C, the DO was sufficiently supplied.

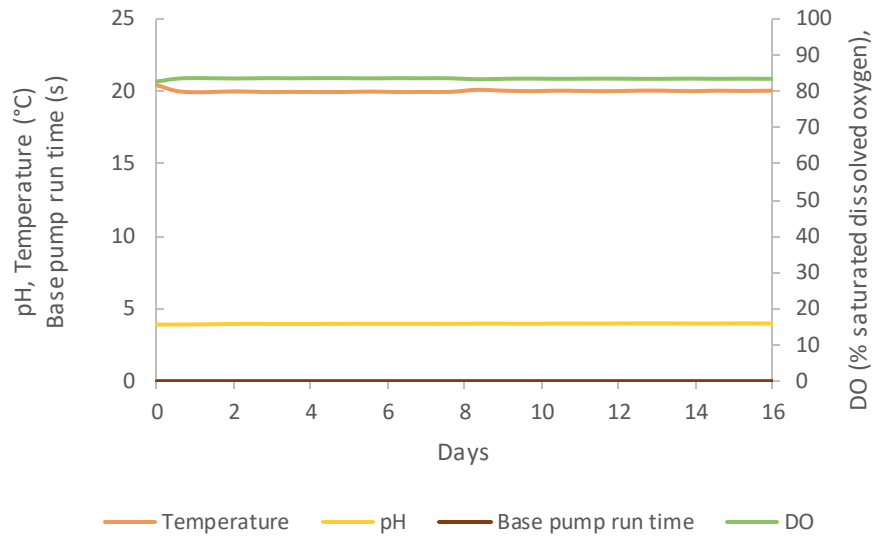


Figure 3.11 Temperature, pH and DO profiles in continuous *M. pulcherrima* fermentation with dilution ratio $1/6 \text{ d}^{-1}$ (HRT 6 days) at 20°C, pH4.

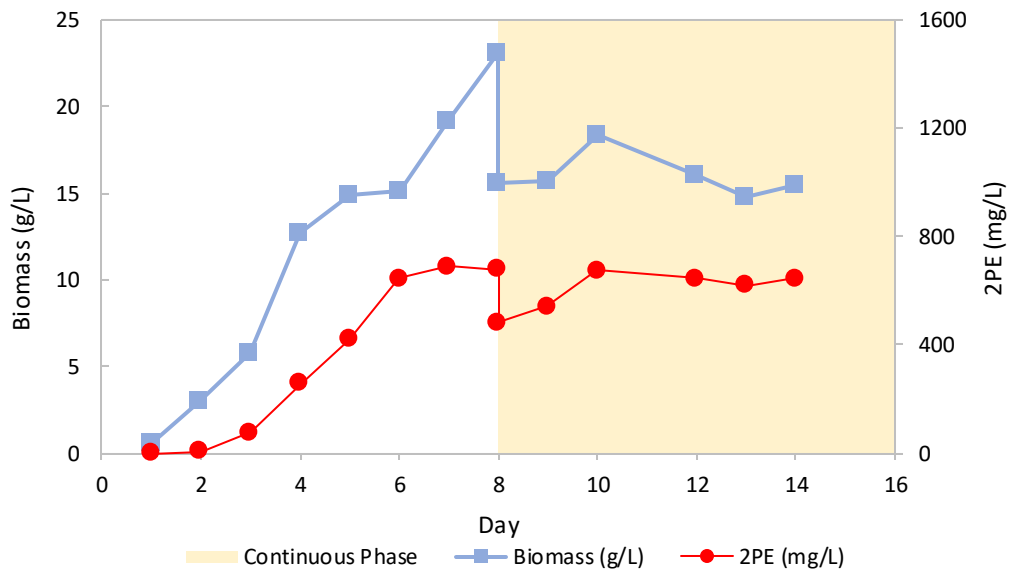


Figure 3.12 Growth and 2PE production from *M. pulcherrima* in continuous bioreactor with dilution ratio $1/6 \text{ d}^{-1}$ (HRT 6 days) at 20°C, pH4. The bioreactor was run in batch mode in the beginning and continuous mode was started since day 8.

In the continuous *M. pulcherrima* fermenter with HRT 6 days, the yeast responded to fresh input and output similar to the dilution rate $1/3 \text{ d}^{-1}$ though to a lesser degree (Figure 3.12), due to the lower dilution rate. Biomass and 2PE were reduced in the first day because of the outflow flux from the bioreactor but the yeast seemed to recover well on the fresh nutrient feed and the biomass started to recover to the previous concentration

seen in the batch growth stage I line with the increased biomass, 2PE production which was reduced from the dilution also recovered to the level observed in the batch running mode. The bioreactor became stable after starting the continuous phase and the biomass was achieved at approximately 15 g/L and 2PE 640 mg/L.

At the lower dilution rate with less new influx media, 2PE was produced at a lower concentration than with HRT 3 days. However, the bioreactor was less prone to flush out. The dilution rate at $1/6 \text{ d}^{-1}$ gave the mean cell residence time 6 days which the culture would normally be in the stationary phase also contributed to the more stable state in the bioreactor.

3.5 Conclusion

2PE production from *M. pulcherrima* was studied on a larger scale in 2L controlled bioreactors, where the effect of aeration could be examined. The yeast scaled well and in the batch mode produced over 700mg/L of 2PE and 14 g/L of yeast biomass under high aeration conditions. It was demonstrated that aeration was one of the important factors that governs the production of 2PE using *M. pulcherrima*. The yeast needs oxygen for maintaining the healthy growth and function properly. The maximum 2PE production in 2L bioreactor was found at the aeration rate of 2 L (1 working volume)/min this productivity was reduced slightly at the 1L/min aeration (620 mg/L), though was hardly produced at lower aeration rates.

The production of 2PE was also achieved in a semi-continuous fermentation with and without biomass being retained to imitate the semi-continuous fermentation with and without settling time before taking out the supernatant in each batch. Both regimes showed a similar pattern of 2PE production which was produced approximately 250-350 mg/L. The lower 2PE than previous experiments may have resulted from smaller working volume and been affected by poor gas transfer or the higher aeration rate that removed the relatively volatile 2PE from the vessels, which can be seen from lower 2PE production but higher biomass production and very high sugar consumption rate. The biomass was naturally higher in the retained biomass experiments.

However, this demonstrated that 2PE could be produced under a continuous mode. To this end a continuous fermentation was undertaken on 2L bioreactors. 2PE production was rapidly improved at the dilution rate of $1/3 \text{ day}^{-1}$ (HRT 3 days) which makes the mean cell age in the growth phase during which 2PE was produced. The advantage of continuous reactor also includes diminished lag phase in the long run and smaller reactor size. 2PE was produced in large quantities by the addition of fresh media which stimulated the yeast activity through the shikimate pathway. Levels reached over 1,500 mg/L in the bioreactor before it became too toxic to the culture and caused the flush out. At the lower dilution rate of $1/6 \text{ day}^{-1}$ (HRT 6 days), the culture was more stable and no flush out was observed. However, the 2PE production was also reduced to 600 mg/L.

M. pulcherrima has demonstrated the possibilities to produce *de novo* 2PE from glucose in single batch higher than any documented in the literature to date and this work also demonstrates that *M. pulcherrima* can be scaled up and produced in different production modes. However, while this is extremely promising, even higher titres can be achieved through the use of phenylalanine as a substrate in the 2PE biosynthesis. In the next chapters the use of this aromatic amino acid as a feedstock will be assessed.

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Chapter 4

2-Phenylethanol Production
via Bioconversion of L-Phenylalanine
using *Metschnikowia pulcherrima*
in 2L bioreactors

4.1 Introduction

In the previous two chapters, *M. pulcherrima* was demonstrated to produce high titres of 2PE, via the *de novo* synthesis route. However, higher titres of 2PE have been reported in the literature from other yeasts through the *ex novo* biochemical conversion of phenylalanine [30].

As the high levels of 2PE can be toxic and slow growth rates, further improvements in 2PE yield can be achieved through the in-situ removal of the resulting 2PE from the fermentation broth. This has the additional benefit of allowing high biomass yields. This was demonstrated by Stark *et. al.* to increase the production 10 fold with a yeast species [46].

In an attempt to increase the yield further, *M. pulcherrima* was therefore cultured with phenylalanine (up to 30 g/L) and the in-situ extraction of the 2PE was examined with both a liquid phase and solid phase absorbent.

4.2 2-PE production by biosynthesis from L-phenylalanine

2-PE is produced in yeast metabolism via Ehrlich pathway with L-phenylalanine as a precursor. In the *de novo* 2PE production, L-phenylalanine is synthesised in yeast from glucose through shikimate pathway, the pathway that can generate aromatic amino acids such as tryptophan, tyrosine and L-phenylalanine from carbohydrate, as shown in Figure 4.1. However, L-phenylalanine production through shikimate pathway in yeast is regulated by the 'general control' mechanisms which responds to the limited supply of amino acids. [81] This means L-phenylalanine production via shikimate pathway will occur only in the condition that the yeast needs amino acids, for example in the growth period where the yeast needs to build new cells. Outside of this condition, the production of L-phenylalanine is limited, resulting in poor 2PE production in the stationary phase of *M. pulcherrima* culture. This was demonstrated to be the case for *M. pulcherrima* in Chapter 2.

However, 2PE production from yeast metabolism will increase greatly if external L-phenylalanine is added to the broth [142, 143]. By providing the L-phenylalanine, the same pathway produces 2PE from this. The possible fate of L-phenylalanine in the yeast metabolism is also shown in Figure 4.1. Therefore, 2PE biosynthesis production with L-phenylalanine in *M. pulcherrima* fermentation could be alternative approach for 2PE production.

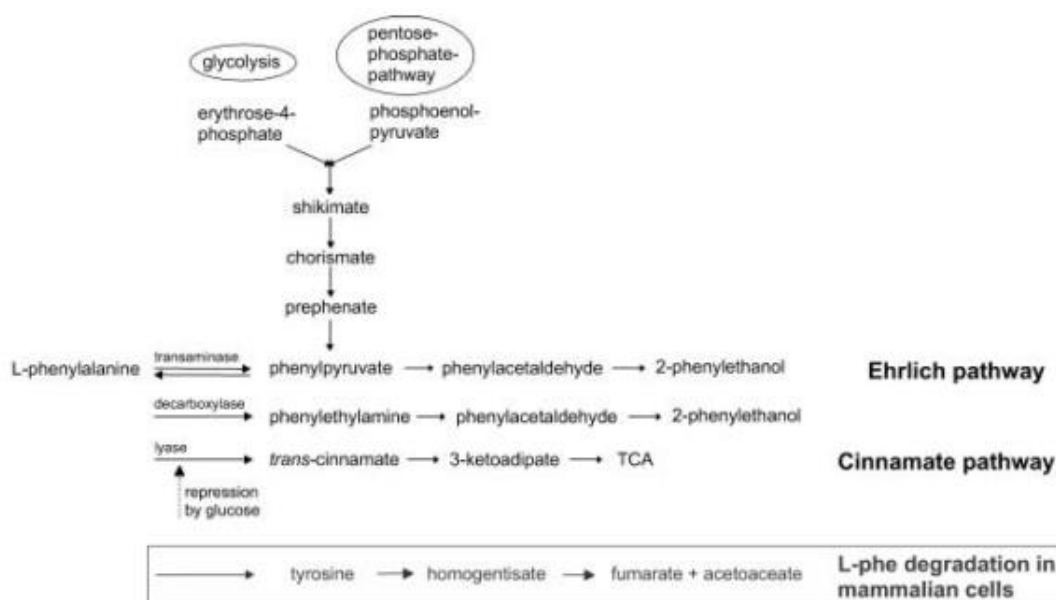


Figure 4.1 L-phenylalanine metabolism through shikimate pathway and 2-phenylalanine production from L-phenylalanine (taken from [30])

The study of 2PE biosynthesis from L-phenylalanine as a precursor by *M. pulcherrima* was firstly done by culturing *M. pulcherrima* in SGJ media with the addition of L-phenylalanine 10, 20 and 30 g/L as a whole nitrogen source. The optimised conditions used to culture the yeast detailed in chapter 2 were used for this study. The fermentation metabolites and glucose utilisation during the fermentation period were analysed by HPLC. The results are shown below in Figure 4.2

The yeast biomass achieved from these cultures were substantially lower than that without phenylalanine, though the growth rate with 10, 20 and 30 g/L of L-phenylalanine was similar to each other. Interestingly, the yeast biomass generally reduced after 3 days in stationary phase down to approximately 2.5 g/L. Small amounts of ethanol were also produced (data not shown) and it is possible that the stress of the large phenylalanine concentration, ethanol production and the large production of 2-phenylethanol all impacted on the yeast biomass production.

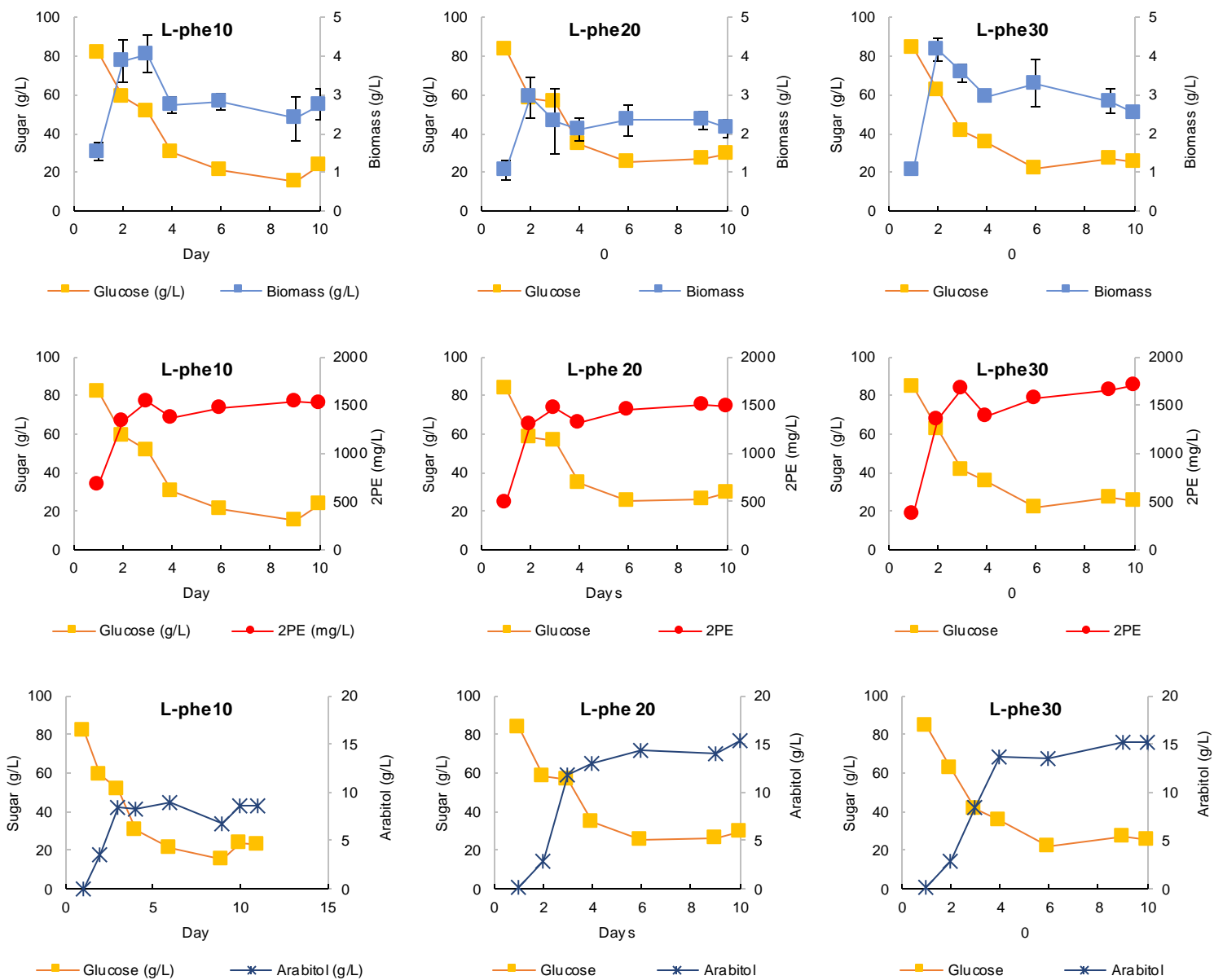


Figure 4.2 Fermentation profile during 2PE biosynthesis from L-phenylalanine 10, 20 and 30 g/L by *M. pulcherrima* in SGJ media pH4 at 20°C

The yeast started to produce 2PE from the first 24 hours and all runs shared the same pattern of the yeast growth. The production reached a maximum during day 3 before becoming stagnating. The maximum production of 2PE of all cultures was between 1,520-1,710 mg/L. As it was seen in Chapter 2, the toxicity limit of 2PE to *M. pulcherrima* is also approximately 1,500 mg/L. This strongly indicates that *M. pulcherrima* can convert 2PE from L-phenylalanine up to the 2PE threshold it can tolerate, approximately 1500-1700 mg/L, presumably the reduction in biomass after this point is due to the 2PE toxicity, coupled with the reduction in biomass is the loss of other metabolic productivity.

The rest of the compound profiles during the fermentation support this conclusion. Glucose was utilised quickly in growth period and even though the yeast stopped growing after day 2, the glucose utilisation continued until day 4, possibly due to the activity of the remaining viable yeast cells, but the uptake completely stopped after that point. The glucose uptake in this experiment is completely different from the previous results where the glucose was usually utilised until the end of the fermentation. The sugar uptake also confirmed the death of *M. pulcherrima* due to 2PE toxicity produced by the biosynthesis.

Arabitol production also demonstrated a similar trend. The production of arabitol stopped after day 3, a day after 2PE was produced exceeding the 2PE tolerance of *M. pulcherrima* in day 2. The arabitol production in the fermentation did not seem to be affected by the added L-phenylalanine, maybe because, unlike *de novo* 2PE or lipid production, arabitol is not regulated by the limitation of nitrogen. The maximum arabitol production in all L-phenylalanine concentrations was about 15 g/L a yield of 0.15 g/g glucose. The arabitol produced in the culture seemed to be excellent for the condition that was not optimised for D-arabitol production from glucose. In comparison, Kiyomoto has patented *Pichia ohmeri* for arabitol production which yielded 0.43 g/g [144]. *Zygosaccharomyces rouxii* was shown to yield 0.48 g/g by [145]. However, the highest conversion yield for arabitol in the literature was produced by *Metschnikowia reukauffii* which yielded 0.52 g/g.

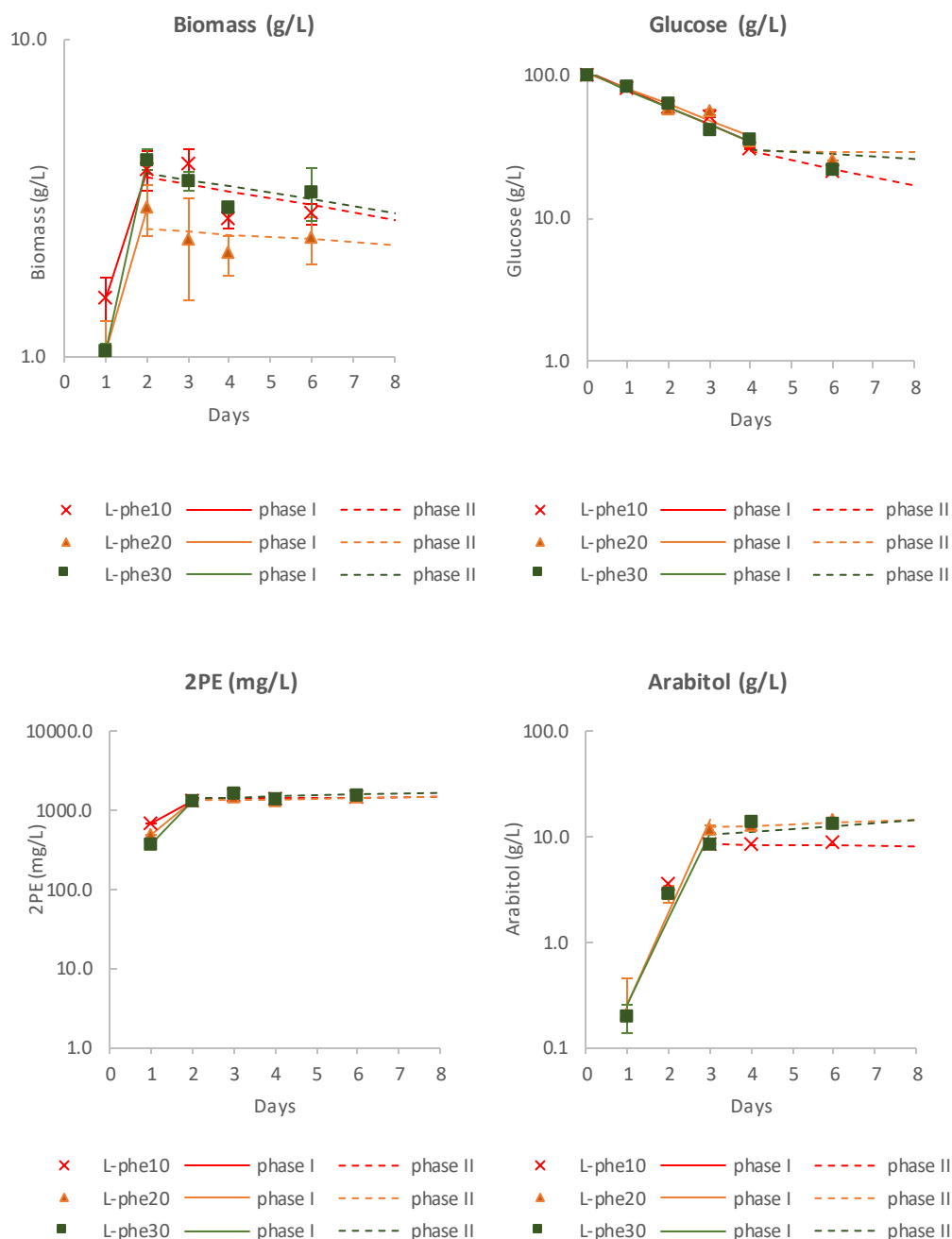


Figure 4.3 Comparison of growth and metabolites productions in the *M. pulcherrima* fermentation with added L-phenylalanine 10, 20, 30 g/L as a whole nitrogen source in SGJ Media pH4 at 20°C, error bars are given as 1 std. deviation (n=3)

The summary of metabolites and residual glucose at the end of fermentation is shown in Figure 4.4. The biomass was adversely affected by high 2PE production from the biosynthesis, and finished with roughly 5 g biomass/L, compared to regular production of 10 g biomass/L. This also affected the rest of the cellular activities including arabinol production and glucose metabolism.

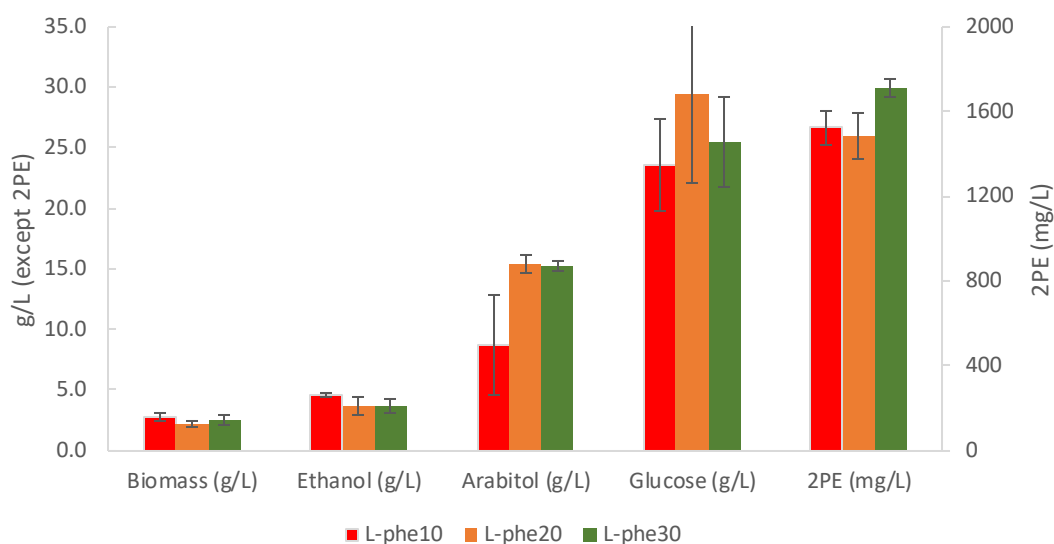


Figure 4.4 End Products in *M. pulcherrima* fermentation with L-phenylalanine 10, 20, 30 g/L as a whole nitrogen source in SGJ Media pH4 at 20°C, error bars are given as one standard deviation (n=3)

2PE, as shown in Figure 4.4, was produced to 1,500-1,700 g/L in all concentrations of 10, 20, 30 g/L. 1g of L-phenylalanine could be converted theoretically to 0.74g of 2PE. The lower achieved yield is likely to be affected by the produced 2PE which killed off the yeast and stopped all the biochemical reaction in the yeast cell.

In order to produce 2PE in the higher amount by biosynthesis, 2PE concentration must be kept under the toxic threshold of *M. pulcherrima* which is approximately 1,500-2,000 mg/L. Therefore, it seems likely that by removing the formed 2PE continually from the reaction into a different phase away from the yeast, then the yield of 2PE could be increased substantially. This has been demonstrated to be successful [142, 146]. The most practical methods for the in-situ extraction process are a liquid-liquid extraction or solid-liquid extraction. In the next section both processes were screened and compared to one another.

4.3 *In situ* liquid-liquid extraction in 2PE biosynthesis production

In a further attempt to improve the production of 2PE an extractant that can separate 2PE from the culture, while the yeast is producing the compounds was examined (in-situ extraction). One of the major difficulties in using in-situ extraction is that the extractant that must have a high affinity to 2PE while will not affecting the growth of the yeast or the 2PE biosynthesis in the yeast.

Two solvents were selected for screening liquid-liquid in-situ 2PE extraction in the biosynthesis; oleyl alcohol (OA) and dodecane (DDC). The solvents were selected due to the properties that can extract 2PE very well and are relatively non-toxic. Firstly, the toxicity of both solvents to *M. pulcherrima* was studied in 25mL culture in 125 mL shaking flasks, incubating at 20 °C for 15 days. The culture was conducted in SGJ media with added L-phenylalanine 2 g/L as a sole nitrogen source. The solvent toxicity was determined by the yeast cell mass (figure 4.5).

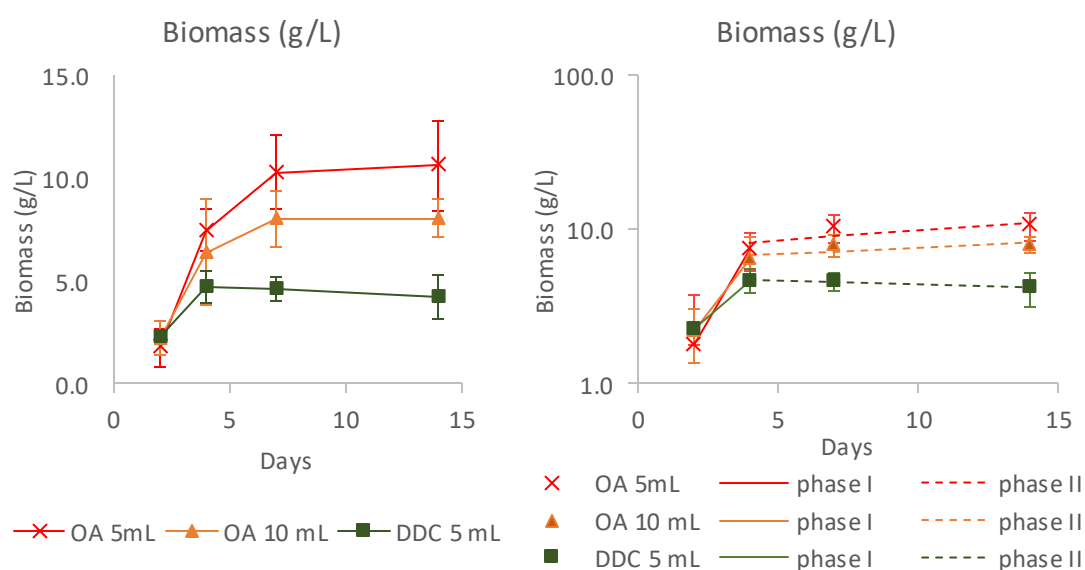


Figure 4.5 Effect of liquid extractants to the growth of *M. pulcherrima* in the 2PE biosynthesis with L-phenylalanine 2g/L as a sole nitrogen source in SGJ media pH4 at 20°C, error bars are given as one standard deviation (n=3)

This demonstrated that *M. pulcherrima* cultures were unduly affected by the extractant added in the mixed culture. Using dodecane as an extractant, yeast biomass was reduced by over half compared to using oleyl alcohol at 5 ml, indicating dodecane is more toxic to *M. pulcherrima* than oleyl alcohol. Oleyl alcohol at 10 ml also demonstrated reduced growth but not as much as dodecane. The inhibitory effect from large levels of oleyl alcohol could be from the cumulative toxicity, if the alcohol is toxic or irritating to the yeast. Alternatively, however, oleyl alcohol affected the aeration of the yeast and limited the oxygen transfer to yeast metabolism. The limited oxygen transfer reduced the oxygen as the electron acceptor in the tricarboxylic acid cycle (TCA), resulted in the less energy production and, consequently, suppressed the growth. As a rough rule of thumb, therefore, the additional oleyl alcohol must not exceed 20% of the total culture volume.

In-situ extraction with oleyl alcohol in 2PE biosynthesis in *M. pulcherrima* was then further studied with the addition of 2 and 5 g/L-phenylalanine in SGJ media with 5mL oleyl alcohol in 25 mL *M. pulcherrima* culture (Figure 4.6). 2PE production in the culture in water phase and alcohol phase was analysed by HPLC. 2PE in oleyl alcohol phase was calculated as mg 2PE produced per culture volume.

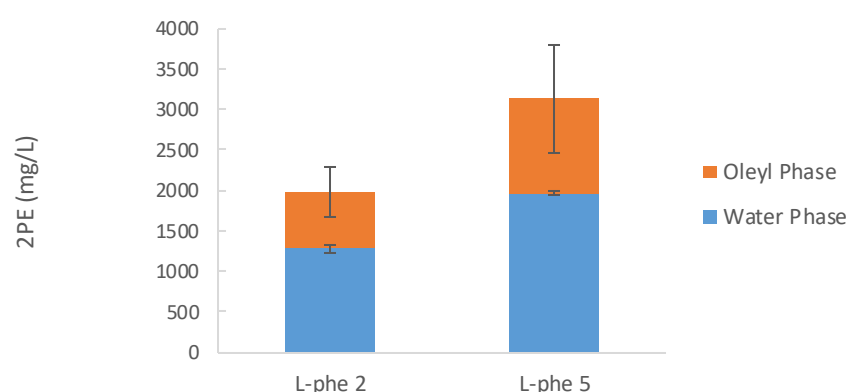


Figure 4.6 2PE production by biosynthesis from L-phenylalanine 2 and 5 g/L with oleyl Alcohol 5mL as an in-situ extractant in *M. pulcherrima* culture 25 mL in SGJ media pH4 at 20°C after 7 days (n=3).

The culture was composed of 2 phases, the water phase and the oleyl alcohol phase. The two phase was mixed to some degree during the shaking in incubating time, but separated easily when left to settle in 30 minutes. 2PE produced in the water phase with added 2 and 5 g/L L-phenylalanine are in the range of 1200-1900 mg2PE/L. This amount of 2PE is similar to the production in the water phase in the previous study with no solvents, suggesting the concentration was governed by the maximum 2PE toxicity threshold to *M. pulcherrima*. However, with oleyl alcohol 5 mL added to 25 mL *M. pulcherrima* culture with L-phenylalanine 2g/L as an in-situ solvent, it yielded 2PE 1.28

g/L in the aqueous phase and 3.5 g/L in the alcohol phase. Similarly, with 5 g/L L-phenylalanine added to the culture, it yielded 2PE 1.97 g/L in the aqueous phase and 5.86 g/L in the alcohol phase.

The total 2PE production is substantially increased up to 1,985 and 3,133 mg 2PE/L for the 2 g and 5 g/L of L-phenylalanine. The produced 2PE in both cultures is approximately similar to the theoretical 2PE percent conversion from L-phenylalanine which is 74% by weight. This suggest that most of L-phenylalanine added was converted to 2PE. *M. pulcherrima* seemed to barely lose L-phenylalanine through the side-pathway such as the cinnamate pathway which will end up wastefully in TCA cycle, but mostly go through Ehrlich pathway and yield 2PE successfully. Though 2PE conversion from 2g L-phenylalanine is slightly higher than theoretical value, the additional amount may be from the parallel de novo synthesis from glucose or simply the error from analytical method.

Similar results with oleyl alcohol as an in-situ extractant was achieved by Etschmann *et al.* (2003) using fermentation with *K. marxianus* in molasses medium 70 mL with 7 g/L L-phenylalanine and 30mL oleyl alcohol and yielded total 2PE 3g/L, increased from 0.8 g/L without the extractant phase [38]. Though, it was less effective than in this study. Other solvents were also experimented such as oleic acid [147] and polypropylene glycol [146] showed similar results.

The slightly higher 2PE production than the theoretical value may be the result from water evaporation during the incubation period or further conversion from the glucose substrate. The results demonstrate that oleyl alcohol is a suitable solvent for the in-situ 2PE biosynthesis production which make 2PE conversion from L-phenylalanine in *M. pulcherrima* efficiently and non-toxic to the yeast.

It has been shown that biosynthesis of 2PE from *M. pulcherrima* can be conducted to increase the production of 2PE by adding a non-toxic solvent in the yeast culture to remove 2PE from the water phase and prevent 2PE accumulation in the water which can be toxic to the yeast. Liquid-liquid extraction with oleyl alcohol is an excellent way to do that. However, even though 2PE can be extracted very well in oleyl alcohol, the separation and purification of 2PE from oleyl alcohol raises further technical issues.

A typical liquid-liquid extraction usually uses a solvent with a low boiling point that can be easily separated from the desired compound by solvent evaporation. However, oleyl alcohol (C₁₈H₃₆O) is non-polar long-chained solvent with a boiling point of 330-360 °C while 2PE has a boiling point at 220°C. The high boiling point of oleyl alcohol, therefore, makes the separation of 2PE from oleyl alcohol difficult and non-economical. On the other hand, the common organic solvents have lower boiling points and easier to

separate from 2PE but they are toxic to the yeast. One possible method to overcome this is to use a solid extractant.

4.4 *In situ* adsorption in 2PE biosynthesis production

Due to the ease of recovery, solid phase adsorbents may provide a better choice for 2PE recovery overall as the 2PE can be recovered from the adsorbent easily with a suitable low boiling point solvent which can be removed by distillation.

Firstly, the use of activated carbon for 2PE recovery from model solutions was compared with other adsorbents such as the zeolite ZSM-5. The study was conducted first conducted in 1000 mg/L aqueous 2PE solution 10.0 mL added with 0.5 g adsorbents (Figure 4.7). The powdered activated carbon (PAC), ZSM-5 were purchased from Sigma and used without further purification. The mixture was agitated at 20°C for 3 days. The remaining 2PE concentration in the aqueous phase was analysed by the HPLC with UV detector at 216 nm.

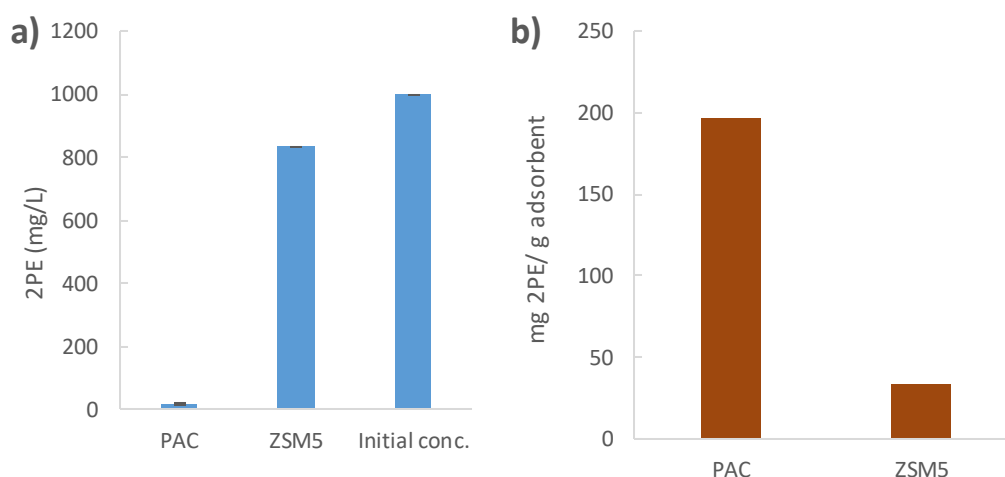


Figure 4.7 2PE adsorption on powdered activated carbon (PAC), ZSM5 in the 10 mL 2PE solution (1000 mg 2PE/L) with adsorbents 0.50 g after 3 days (a) 2PE concentration in the aqueous phase after adsorption (b) adsorption capacity 2PE on the adsorbent

PAC was demonstrated to be an extremely effective adsorbent of 2PE, with only trace levels left in the aqueous phase (Figure 4.7). In contrast, ZSM-5 can absorb 2PE to a small degree, 830 mg/L of 2PE was left in the aqueous phase from the initial 2PE concentration of 1000 mg/L. The calculated 2PE capacity in ZSM-5 was therefore only 33mg 2PE/g adsorbent in this experimental set up where was at least 200 mg 2PE/ g adsorbent for PAC. The large 2PE adsorption capacity of PAC showed that PAC can be an excellent adsorbent in the in-situ 2PE biosynthesis fermentation.

At the equilibrium with an adsorption capacity at 200 mg/g, 2PE concentration was left as low as 18 mg/L may suggest the maximum adsorption capacity of PAC could be higher. Therefore, the 2PE adsorption characteristic and isotherm equilibrium was further examined by varying the concentration of 2PE aqueous solutions with PAC 0.1g as an adsorbent. The adsorption conditions were the same as previously used. 2PE in the aqueous-phase (C_e) and in the adsorbed-phase (q_e) were then calculated and plotted.

$$q_e = \frac{(C_i - C_e)}{m_{pac}} \times V_s$$

, where q_e = adsorbed 2PE fraction on PAC (g/g)

C_i = initial concentration of 2PE (mg/L)

C_e = equilibrium of 2PE (mg/L)

m_{pac} = mass of PAC (mg)

V_s is the volume of solution (L)

The adsorption was determined using the Langmuir isotherm, plotting the C_e/q_e and C_e according to the correlation for Langmuir isotherm. The plot (Figure 4.8) shows that 2PE adsorption on PAC fits the Langmuir isotherm ($r^2 = 0.9928$). The maximum Langmuir adsorption capacity (q_{max}) is 0.807 g/g.

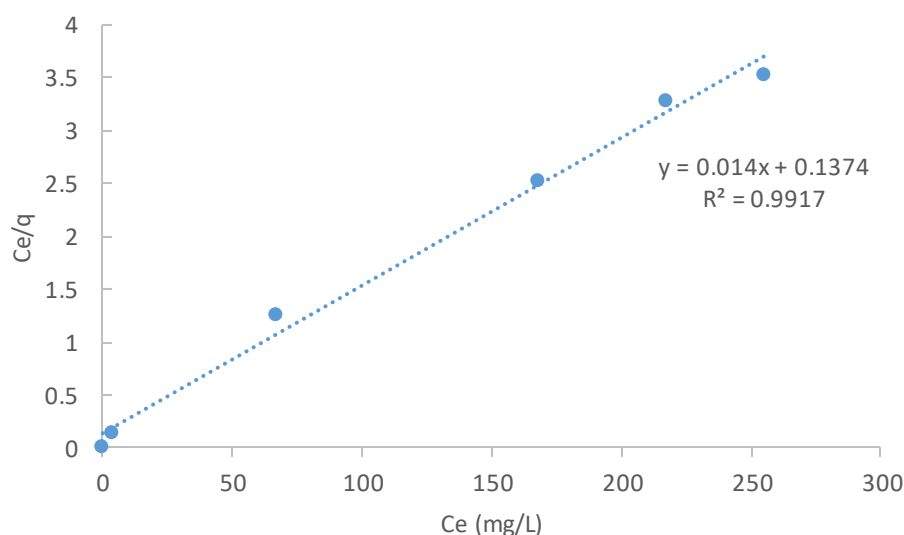


Figure 4.8 Langmuir isotherm plot of 2PE adsorption on powdered activated carbon (PAC)

Activated carbon is originally charcoal but chemically and thermally treated to be a highly porous carbon material and has a high surface area, typically more than $1000 \text{ m}^2 \text{ g}^{-1}$. The extensive surface area makes activated carbon potentially with enormous adsorption capacity. In addition, activated carbon is a non-toxic material and widely used in adsorption application with plants, animals and human use.

Due to the excellent 2PE absorption demonstrated by PAC, the absorbent was then added to cultures of *M. pulcherrima*. The cultures were grown on SGJ media 25 mL with L-phenylalanine 10, 20 and 30 g/L as a precursor to the 2PE biosynthesis in Erlenmeyer flasks. PAC 0.5 g was used. The culture was incubated at 20°C for 7 days. Biomass could not be measured due to the interference from PAC. However, on completion, the cultures were centrifuged at 6,000 rpm to separate the aqueous phase. The centrifuged biomass and PAC were leached by 5mL methanol twice. The extract was then analysed by HPLC with UV detector at 216 nm.

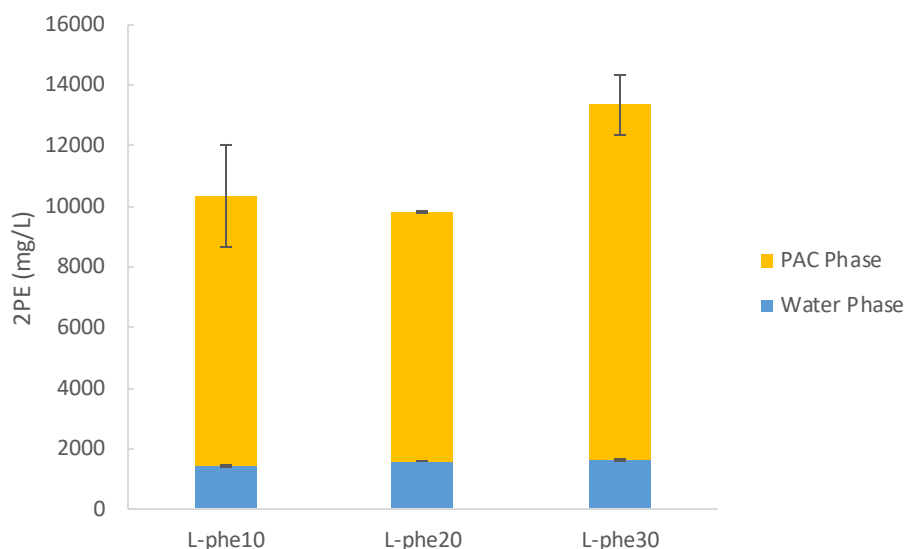


Figure 4.9 2PE production by biosynthesis from L-phenylalanine with powdered activated carbon (PAC) as an in-situ extractant in *M. pulcherrima* culture 25 mL in SGJ media pH4 at 20°C after 7 days ($n=3$)

2PE production by *M. pulcherrima* with PAC was demonstrated to be increased dramatically, as shown in Figure 4.9. 2PE in the aqueous phase is approximately 1,500 mg/L in all L-phenylalanine concentrations, similar to the previous experiments. However, 2PE in the solid phase which was adsorbed on PAC has increased greatly, ranged from 7 to 10 g 2PE/L. In the culture with 10 g/L L-phenylalanine, 2PE was produced 7.4 g/L, showing that the yield is corresponded exactly to the theoretical yield (theoretical yield of 2PE production 0.74 g/g L-phe), suggesting *M. pulcherrima* can convert all L-phenylalanine to 2PE without any genetic modification, provided 2PE is kept lower than the toxic threshold to the yeast. PAC 0.5 g can sufficiently adsorb produced 2PE 7.4 g/L in the culture condition, owing to its large adsorption capacity.

In the *M. pulcherrima* culture with L-phenylalanine 20 and 30 g/L, the 2PE produced was not increased by the increasing amount of the precursor, with yields of only 0.332 and 0.337 g/g phenylalanine, respectively. This indicated that PAC had adsorbed 2PE to its full capacity under these culture conditions, with the excess 2PE production inhibiting further 2PE conversion. Therefore, the 2PE production was still limited. However, considered PAC was added 0.5 g/ 25 mL culture, PAC could be added more to the culture to remove 2PE from the culture, preventing the inhibitory effect to the yeast. For practical purposes, a rough estimate of 0.5 g PAC may be suggested for each 10 g L-phenylalanine added in the biosynthesis.

M. pulcherrima with activated carbon as an in-situ adsorbent was shown to be an excellent 2PE production system for the bioconversion from L-phenylalanine, at the maximum 14 g 2PE/L was demonstrated. This is on par with 2PE production using Hytre® by Gao and Daugulis (2009) which can produce 2PE 13.7 g/L in batch culture

and is claimed to be the highest among literature [47]. In comparison, Etschmann and Schrader reported 2PE production of 10.2 g/L from *K. marxianus* using polypropylene glycol 1200 [146], Hua et al. (2010) used the hydrophobic polystyrene resin HZ818 and showed 6.6 g 2PE/L was produced from a *S. cerevisiae* fermentation.

The final aspects of study in using PAC as an in-situ 2PE removal is 2PE recovery from PAC. As previously stated, one of the advantages of using PAC as an adsorbent over liquid-liquid extraction is the ease of 2PE recovery. This can be done by leaching the adsorbed 2PE into a suitable solvent and then evaporating the solvent, yielding purified 2PE. Therefore, the selection of solvents for leaching could be vital to the 2PE biosynthesis from L-phenylalanine.

In the solvent selection two properties are desirable which are the 2PE leachability and the ease of evaporation of the solvent. The ideal solvent should show the highest 2PE affinity and has the lowest boiling point. The 2PE affinity of the solvents was studied by the adsorption of 2PE at equilibrium with the solvents where 2PE 10 mg and PAC 0.1 g was added to the solvents 10 mL. The mixture was incubated at 20°C overnight and was diluted and filtered prior to 2PE analysis by HPLC with UV detector at 216 nm.

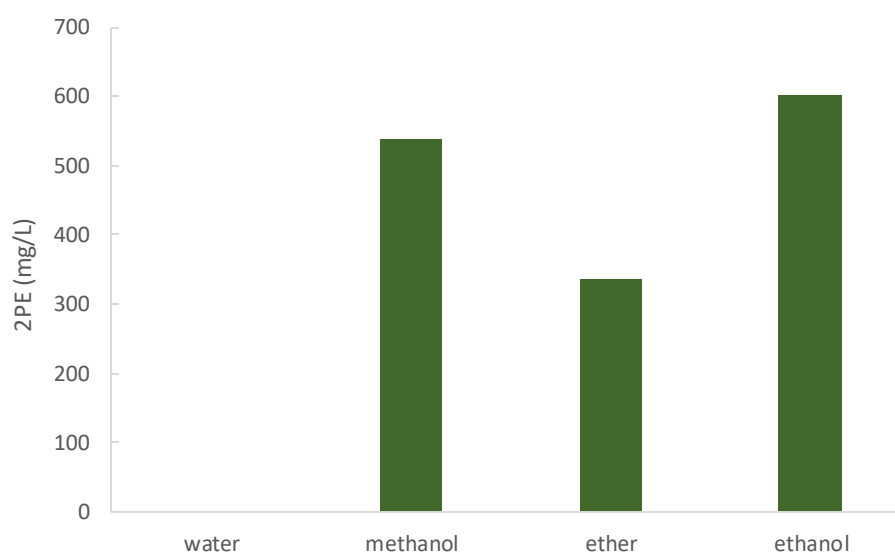


Figure 4.10 2PE concentrations at equilibrium in different solvents with 2PE 10 mg and PAC 10 mg in 10mL of solvents in leachability study

Methanol and ethanol have been shown to be good solvents because of their 2PE high leachability (fig. 4.10). 2PE can be leached by ethanol by 60%, slightly better than methanol (53%) and far better than diethyl ether (33.5%) under the conditions tested. The boiling points of these solvents are relatively low, diethyl ether (34.6°C), methanol (64.7°C) and ethanol (78.4°C). Even though that ethanol may show a better leachability,

methanol may also be selected for 2PE leaching as the leachability is not far behind and boiling point is lower, making it easier to separate from 2PE by evaporation.

4.5 Conclusion

M. pulcherrima was demonstrated to be able to convert L-phenylalanine to 2PE via the in-situ biosynthesis (theoretical yield 0.74 g 2PE/ g L-phenylalanine), in addition to the de novo 2PE production from glucose. However, high 2PE concentration can inhibit 2PE production from the yeast. The threshold concentration for *M. pulcherrima* is approximately 1,500-1.700 mg/L. If higher 2PE production is preferred, the in-situ 2PE removal such as liquid-liquid extraction and solid adsorption is needed to be introduced into the culture to prevent the inhibitory effect from high 2PE production.

Oleyl alcohol was found to be a suitable solvent for liquid in-situ 2PE removal as it is non-toxic to the yeast and can extract 2PE very well. However, the addition of high amounts of the solvent was shown to reduce the yeast productivity. Also the disadvantage of liquid-liquid extraction is the difficulty of removing the 2PE from the solvent-phase.

Powdered Activated Carbon (PAC) was selected for in-situ 2PE removal in *M. pulcherrima* culture. It has demonstrated the high 2PE capacity and can increase the 2PE production up to 10g/L with added PAC 2% w/v. The 2PE recovery was effective with either methanol or ethanol as a leaching solvent from the adsorbed PAC.

4.6 References

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Chapter 5

2-Phenylethanol production from the
bioconversion of phenylalanine
using *Metschnikowia pulcherrima*
in 2L bioreactors

5.1 Introduction

Two-phase partitioned bioreactor (TPPBs) are bioreactors that have an additional non-aqueous phase (NAP) added to the bioprocess to limit the inhibitory effect from chemicals which are either in the substrate or metabolic products. In environmental applications, they are mostly used on the degradation of highly toxic compounds which can be adsorbed in the NAP and slowly released into the aqueous phase, making the concentration to the microbial community tolerable [148]. In fermentation applications, this type of bioreactor is used where the target product can be inhibitive [30, 142, 149]. Specifically, when used with citric acids or ethanol it is sometimes called 'extractive fermentation' [150-153]. The NAP itself can be either non-soluble liquid [46] or a solid phase [154, 155].

As demonstrated in previous chapters, 2PE displays some inhibition towards *M. pulcherrima* at a concentration of 1,500 mg/L or above, where it was shown to limit growth substantially. The conversion of L-phenylalanine has been demonstrated to be so efficient under optimal conditions, that this toxic concentration could be achieved easily when the level of L-phenylalanine is high enough. For example, a total production of 2PE, was achieved at 10g/L only when the concentration at any point in the aqueous phase was kept under 1500 mg/L. In the scaling-up of the process into bioreactors, a TPPB approach is highly suitable for 2PE production.

The non-aqueous phase extractants in TPPBs can be selected to suit the fermentation and adsorbates in the reactors, and various examples are presented in the literature, such as oleic acid [46], oleyl alcohol [156] and polypropylene glycol 1200 [146]. In the previous chapter it was demonstrated that oleyl alcohol and activated carbon were highly suitable for 2PE extraction in *M. pulcherrima* cultures grown in shake flasks. Both extractants were therefore used as the NAP in this experiments.

Oleyl alcohol, which is a liquid extractant, can be used as NAP in fermentation broth without interfering with the yeast's activities. However, the disadvantage is a lower capacity to dissolve 2PE and the difficulties in removing the 2PE from the oleyl alcohol once the reaction is finished. Activated carbon, on the other hand, provided a better choice because of the higher capacity and the separation can be easier achieved by leaching 2PE-adsorbed activated carbon with a suitable solvent. However, activated carbon is not without its drawbacks. It was observed in the previous chapter that the culture with PAC is more prone to contamination, possibly because activated carbon adsorbed the biocontrol chemicals the yeast produced to keep off the contamination as well as 2PE, which make it lose the natural mechanism to fight off the invading contamination in the less sterile conditions.

Activated carbon is an inert highly porous charcoal that is activated to create numerous pores on the surface of the coal. The created mesopores and micropores resulted in very high surface area per volume, usually more than 1000 m²/g [157, 158]. Its high surface area makes activated carbon one of the most popular adsorbent used in many applications including medical applications, water purification, industrial separation and catalysis, environmental remediation, and fuel storage [159-161].

In this chapter the process for 2PE biosynthesis from L-phenylalanine using *M. pulcherrima* was tested in a two-phase partitioning bioreactor, using the optimal conditions presented in the last chapter with activated carbon used as the extractant. The bioconversion of 2PE from L-phenylalanine by *M. pulcherrima* in 2L bioreactor using activated carbon was examined in different reaction modes including batch mode, continuous mode and semi-continuous mode.

5.2 Activated carbon sizing for 2-Phenylethanol production from *M. pulcherrima* in a two-phase partitioning bioreactor

The experiment on 2PE production from L-phenylalanine bioconversion in two-phase partitioning bioreactor was conducted in a 2L bioreactor, pH4 and 20°C. The aeration rate was kept at 2L/min (1 working volume/min) and agitated at 150 rpm.

In the previous chapter, the powdered form of activated carbon was used in the shake flasks, this was purchased from Sigma Aldrich and had a particle size of 100 mesh. The small particle size of the activated carbon available, increased contact with the 2PE produced by the yeast, however, its small powdered size made it well suspended in the media and the particles did not settle easily. While this was ideal for the shake flask experiments and it excelled in the adsorption, its longer settling time and tendency to be resuspended, if agitated, may not be favourable in the following separation process. In the adsorption column, the small particle size also made it less permeable and not suitable for the process, both in 2PE adsorption and leaching. In the larger size form, activated carbon performs better in column process as it has a higher permeability and is less likely to clogged, but too larger size can reduce 2PE adsorption capacity resulting in 2PE in the solution leaving the column before it can diffuse to activated carbon adsorption sites, due to its larger size.

In the in-situ TPPB, it is also likely that the yeast will grow on the activated carbon, blocking the adsorption diffusion which would be worse in the larger particle size of activated carbon. This has been observed in similar fermentations [162-165]. Concerning the proper size of activated carbon in bioreactor fermentation, the 2PE adsorption and leaching experiment on different sizes of activated carbon was studied.



Figure 5.1 Activated carbon in various size, from left to right, powdered, GAC2040 (20-40 mesh), GAC0412 (4-12 mesh) and commercial activated carbon

In the study of 2PE adsorption on different size of activated carbon, the adsorbent was used in 4 different sizes, powdered, granular Draco 20-40 mesh, granular Draco 4-12 mesh, which are all supplied by Sigma Aldrich and granular commercial activated carbon Fluval Carbon, purchased from Amazon UK, typically used in household water purification equipment. The adsorption study used an initial 2PE aqueous solution of 1000 mg/L and added activated carbon 1g / 50 mL solution in 125 mL Erlenmeyer flasks, shaking at 20°C and 180 rpm. The experiments were done in triplicate for each size. The samples were collected from the aqueous phase after 5 min, 10 min and 2 hours and analysed for 2PE.

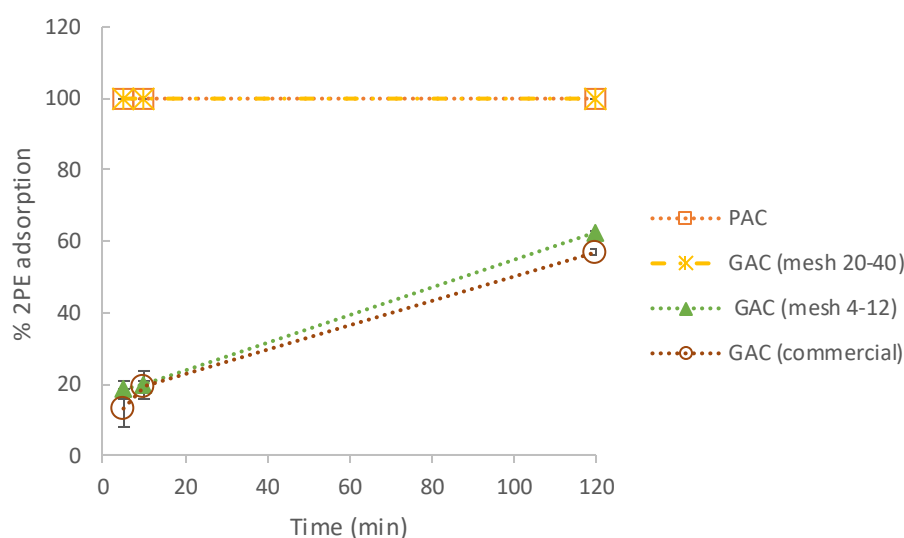


Figure 5.2 2PE adsorption on different sizes of activated carbon in shake flasks with initial concentration 1000 mg 2PE/L and adsorbents 1 g/50 mL solution at 20°C, 180 rpm. The error bars represent one standard deviation ($n=3$)

PAC and the small size granular activated carbon GAC 2040 (20-40 mesh) performed very well in the 2PE adsorption test (Figure 5.2). The adsorption occurred rapidly on these carbon adsorbents and the 2PE was completely adsorbed within 5 minutes. This suggests that GAC2040 can perform reasonably on par with PAC and could replace PAC in the process.

The adsorption rate is slower in the larger size granular activated carbon GAC0412 (4-12 mesh). It only adsorbed 20% of the 2PE in the first 5 minutes and did increase this substantially until after 2 hours, where 62% of the 2PE was absorbed. The adsorption of 2PE on the commercial GAC, which has a size similar to GAC0412, was slightly below GAC0412. The adsorption capacity of the larger granular activated carbon was far less than anticipated though it is unclear what caused this worse performance.

As previously discussed, powdered activated carbon have been the best in adsorption rate as the minuscule particle makes 2PE diffuse easily into the pore sites, but it

presented problems in the involving processes such as adsorption column and separation unit. On the other hand, GAC2040 which is a relatively small-size granular activated (20-40 mesh) has shown a good adsorption character, not substantially different from PAC and provided a relatively higher permeability and settling time. Therefore, GAC2040 was selected instead of PAC, which had been used in the previous chapter, in the study of 2PE bioconversion of *M. pulcherrima* in the TPPB both in the in-situ batch bioreactor and ex situ continuous bioreactor. Two-phase partitioning bioreactor with *in-situ* activated carbon

M. pulcherrima was cultured in the TPPB with activated carbon as an *in-situ* extractant and controlled in batch mode. The media for bioconversion was SGJ media with the addition of 20g/L of L-phenylalanine. Activated carbon (GAC2040, 20-40 mesh) was sterilised and added directly in the bioreactor in steps of 20 g or 50 g when 2PE in the aqueous phase was over 500 mg/L. The purpose of stepped adding GAC was to trace 2PE concentration in the bioreactor which could not be traced if GAC was overloaded and adsorb all the traceable 2PE produced. The threshold of 500 mg/L was selected as the requirement for adding new activated carbon. The maximum activated carbon was 150 g.

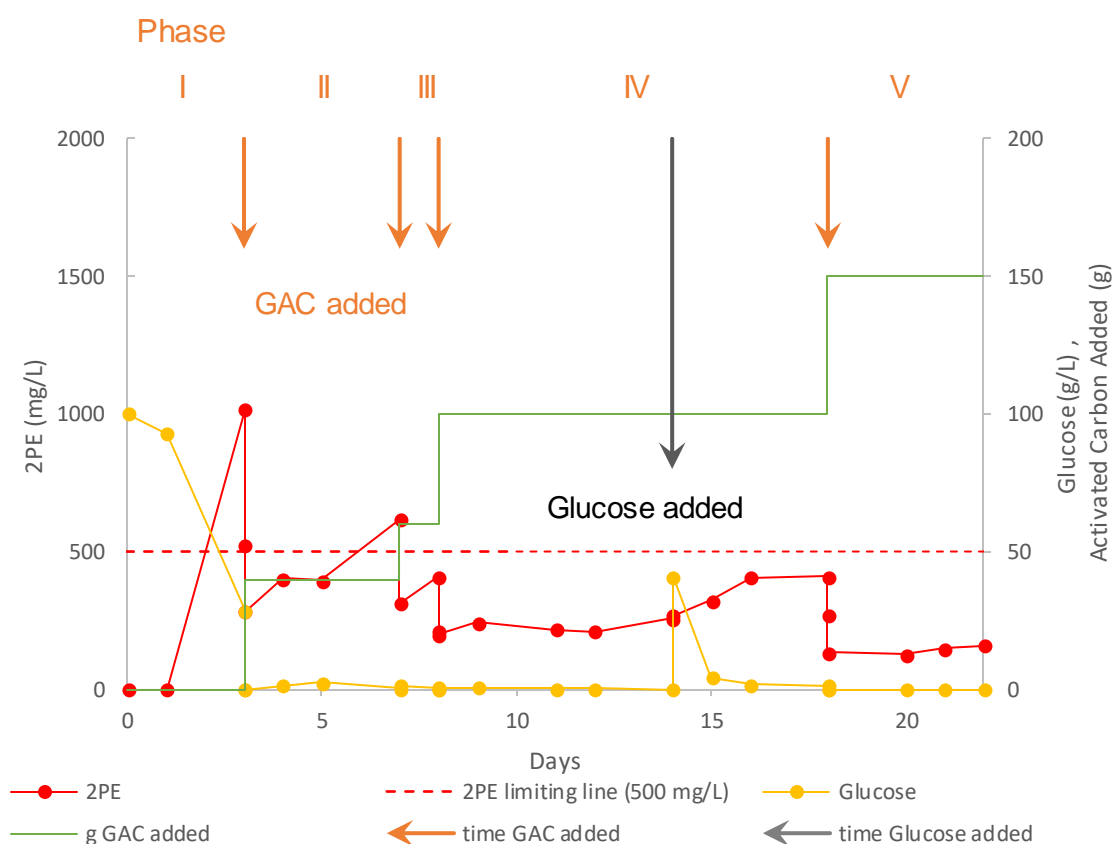


Figure 5.3 2PE production in solid-liquid two-phase partitioning bioreactor with stepped adding granular activated carbon (GAC2040, mesh 20-40), cultured in SGJ media with added L-phe 20 g/L at pH4, 20°C with aeration 2L/min. 2PE shown is 2PE concentration in the supernatant phase.

Along the time of fermentation, activated carbon was added several times when 2PE concentration was over 500 mg/L. In the first phase, the yeast grew normally and started to produce 2PE on day 2. GAC was not added to the culture in this phase to trace if the yeast continued to grow, as the biomass cannot be measured in the TPPB because the suspended GAC in the culture can interfere with the optical density and biomass measurement. Also, the yeast can grow as a biofilm on GAC and would not be reflected by the optical density of the culture, which is how the biomass is determined. In the first phase, 2PE was produced at over 1,100 mg/L from the first few days. GAC (20-40 mesh) was added 20 g (10g/L) in day 3 to prevent 2PE in the aqueous phase reaching the inhibitory concentration (1500 mg/L). After this was added, 2PE concentration was still over 500 mg/L, therefore another load of GAC (20 g/L) was added. After the total GAC in the system was 40g (20g/L), 2PE was left in the aqueous phase at approximately 250 mg/L and the adsorbed 2PE on GAC was calculated to be 850 mg/L. The adsorbed 2PE on GAC was 42.5 mg 2PE/ g GAC. The adsorbed amount was lower than expected because the maximum adsorption capacity of activated carbon was shown to be over 800 mg 2PE/g AC in the previous chapter. The reduced adsorbed capacity of the adsorbent could be due to the reduced pH of the fermentation.

In the second phase (day 3-7), 2PE was continuously produced after the added activated carbon, however the production rate was seen in the aqueous phase as slower than in the first phase where there was no adsorbent, because 2PE produced by *M. pulcherrima* by bioconversion from L-phenylalanine was adsorbed on activated carbon to keep 2PE in the culture lower than the toxic concentration. Another 20 g GAC was added in day 8 to keep 2PE below 500 mg/L again. At this point in the culture there was no glucose left in the bioreactor.

Another GAC 40 g was added at day 8 after 2PE was increased during phase 3. However, in Phase 4, 2PE production seemed to reduce. This reduction in production was presumably down to a shortage of either glucose or phenylalanine. While the HPLC demonstrated that there was L-phenylalanine left in the reasonable amount in the culture, therefore, it could be because of the shortage of glucose. At this point glucose was added to test this hypothesis. After adding glucose 40 g/L on day 14, 2PE was produced again. It emphasised the importance of glucose and suggested that glucose must be available during the fermentation for 2PE bioconversion even though the exponential growth is not expected this late in the fermentation. The experiment was terminated on day 22 with a total production of 8g of 2PE from 20g of phenylalanine in the initial media. This is a conversion of 54% of the theoretical maximum, though it should be noted that a large proportion of glucose was added and *de-novo* synthesis would also have taken place.

5.3 Attempts on continuous two-phase partitioning bioreactor with ex-situ activated carbon

In the 2PE production study from *M. pulcherrima* by bioconversion from the amino acid L-phenylalanine, it was previously demonstrated that the yeast can convert the amino acid to 2PE as long as 2PE was kept lower than an inhibitory concentration of approximately 1,500 mg/L which was achieved in the batch bioconversion by adding in-situ activated carbon as an extractant throughout the 22 day fermentation. However, the batch process has some disadvantages such as the requirement of the time and carbon source for biomass building up in the early stage of the growth period in the fermentation.

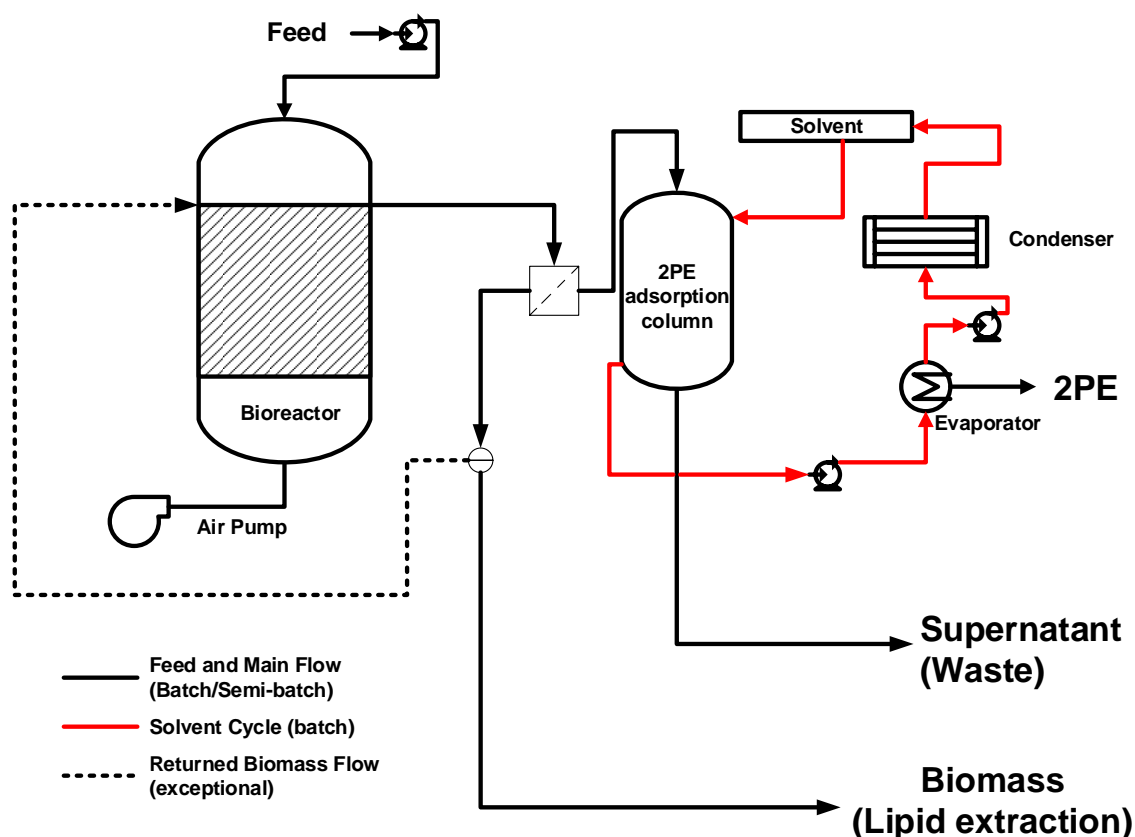


Figure 5.4 Plan for continuous bioreactor with ex-situ activated carbon adsorption column and 2PE leaching cycle

While 2PE bioconversion from L-phenylalanine is not dependent on the growth period, it would need to create biomass to be sufficient for the conversion. The fermentation could be benefit if the time and the substrate was reduced by another regime of fermentation. Therefore, a continuous bioreactor for 2PE bioconversion was also investigated using activated carbon as an extractant in an external column to prevent the flush out of the activated carbon (Figure 5.4).

The input feed was fed directly into the bioreactor and the output was flowed through the adsorption column with GAC0240 (20-40 mesh) as the adsorbent. The 2PE was removed by the adsorbent and the rest of the stream sent to waste. When the adsorption in the column was at its limit, methanol was used as the solvent to leach 2PE from the column. Even though ethanol has also been used to extract 2PE [38], methanol was used because of its lower boiling point. The 2PE was easily recovered from the methanol using simple evaporation of the solvent.

A cross flow membrane was introduced to the overflow output to separate the biomass from the output stream allowing the aqueous phase, with 2PE, to pass through in the permeate and the yeast-rich retentate was returned to the bioreactor to maintain the biomass. The biomass in the bioreactor was controlled by direct removal to maintain a steady concentration. The yeast biomass can potentially then be used for further products such as lipid or protein.

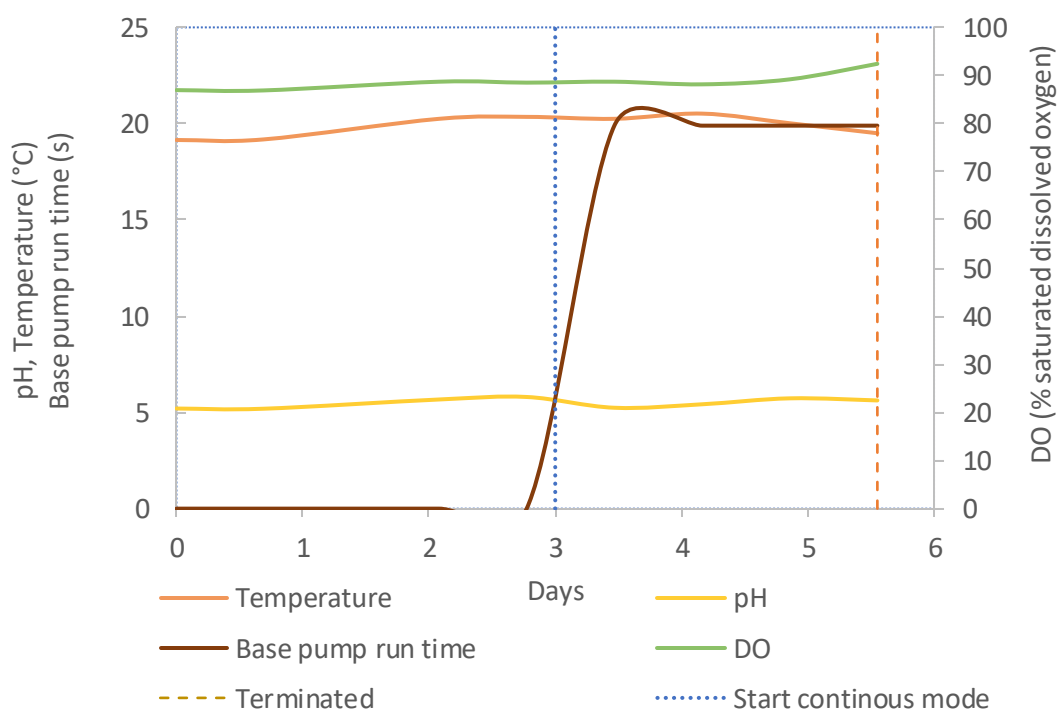


Figure 5.5 Continuous 2PE biosynthesis by *M. pulcherrima* fermentation in 2L bioreactor with SGJ media with L-phe 20 g/L at pH4, 20 °C with granular activated carbon as an ex-situ 2PE removal unit. Continuous feed started at day 3 with dilution ration 0.33 day⁻¹. The fermentation was terminated at day 5 due to contamination. .

The experiments with granular activated carbon in column as an ex situ 2PE removal unit was conducted in the biosynthesis fermentation with L-phenylalanine as a precursor in 2 modes. The first experiment connected activated carbon column where the outflow from the reactor was flowed through the column. The parameters in the fermentation were shown in Figure 5.5. However, the column had tendency to clogged and needed

backwashing. However, the major problem in this set up was the contamination which led to fermentation termination in day 5.

The second continuous 2PE biosynthesis with granular activated carbon column as an ex situ 2PE removal, an additional cross-flow membrane unit was installed to separate yeast biomass in front of the adsorption column to prevent the clogging. The fermentation parameters were shown in Figure 5.6

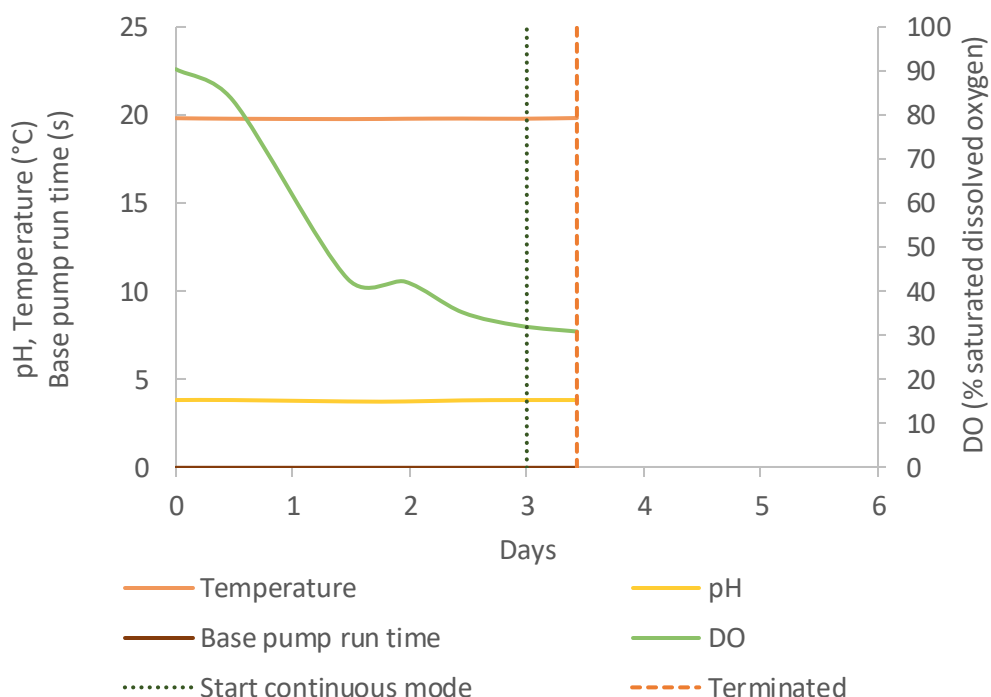


Figure 5.6 Continuous 2PE biosynthesis by *M. pulcherrima* fermentation in 2L bioreactor with SGJ media with L-phe 20 g/L at pH4, 20 °C with granular activated carbon as an ex-situ 2PE removal unit and cross-flow membrane unit. Continuous feed started at day 3 with dilution ratio 0.33 day⁻¹. The fermentation was terminated at day 5 due to contamination. .

Unfortunately, contamination which is controlled on the batch process by the yeast effectively, is a critical problem in the continuous system, even when the reactor and feed were sterilised prior to the runs. This is presumably due to the external flow through the adsorption column being exposed to non-sterile conditions. It seems likely that while *M. pulcherrima* can produce antibacterial compounds these were adsorbed on the activated carbon throughout the experiment. Indeed, 2PE is one of these control agents and its continual removal will not aid the production. Another mechanism that *M. pulcherrima* controls contamination is through iron depletion by producing an iron binding agent; pulcherriminic acid. This prevents other microorganisms from accessing the iron. It is possible that the iron impurities in activated carbon can impair the iron depletion biocontrol mechanisms as well.

Even though the continuous bioreactor with the ex situ activated carbon adsorption was a promising regime for 2PE conversion from L-phenylalanine with many advantages, the difficulty in the counter contamination was a challenge rendering this system ineffective.

5.4 2-Phenylethanol production from *M. pulcherrima* in a semi-continuous bioreactor

Further study of 2PE production from the bioconversion of L-phenylalanine using *M. pulcherrima* was conducted in the semi-continuous fermentation in the 2L bioreactor. Due to contamination of the continuous system, it was reasoned that a semi continuous system would allow the build-up of antibacterial compounds, but the safe removal of 2PE before it rose to toxic levels.

M. pulcherrima was cultured in SGJ media with L-phe (1 g/L) in the 2L bioreactor. The fermentation could proceed until the yeast was in the exponential phase. At this point the semi-continuous process was started with a dilution ratio of 0.5. On removal of 1L of culture, fresh SGJ media with 2 g/L L-phenylalanine was refilled into the fermenter and the fermentation continued. Samples were collected to trace the growth and 2PE production. L-phenylalanine was estimated by directly analysis with HPLC with UV detector at 216 nm. The profile of fermentation is shown in Figure 5.7.

The yeast grew well in the first phase and reached stationary phase by day 7. 2PE was observed from day 4. Both the lag time and the production of 2PE were slower than in other experiments, possibly due to the lack of sterility in the feed. In day 7, the semi-continuous process was started at a dilution ratio of 0.5 where half the culture was taken out and replaced with the new medium with L-phenylalanine 2 g/L.

The culture was replaced with new media in day 8 as the growth rate from previous experiments demonstrated this would be a stable system. However, the culture remained stagnant and only sluggish production of 2PE was observed. By day 20 the yeast had recovered and the culture was replaced with the new medium by dilution 0.5 in day 20. 2PE was produced in very high titres after this point with the system producing 1000 mg/L after 2-3 days, for the remaining 8 days the production of 2PE was produced steadily in this semi continuous fashion. On stabilisation of the reactor, *M. pulcherrima* can synthesise 2PE for approximately 900 mg/L from L-phenylalanine 2 g/L which suggested complete conversion with 0.5 dilution ratio.

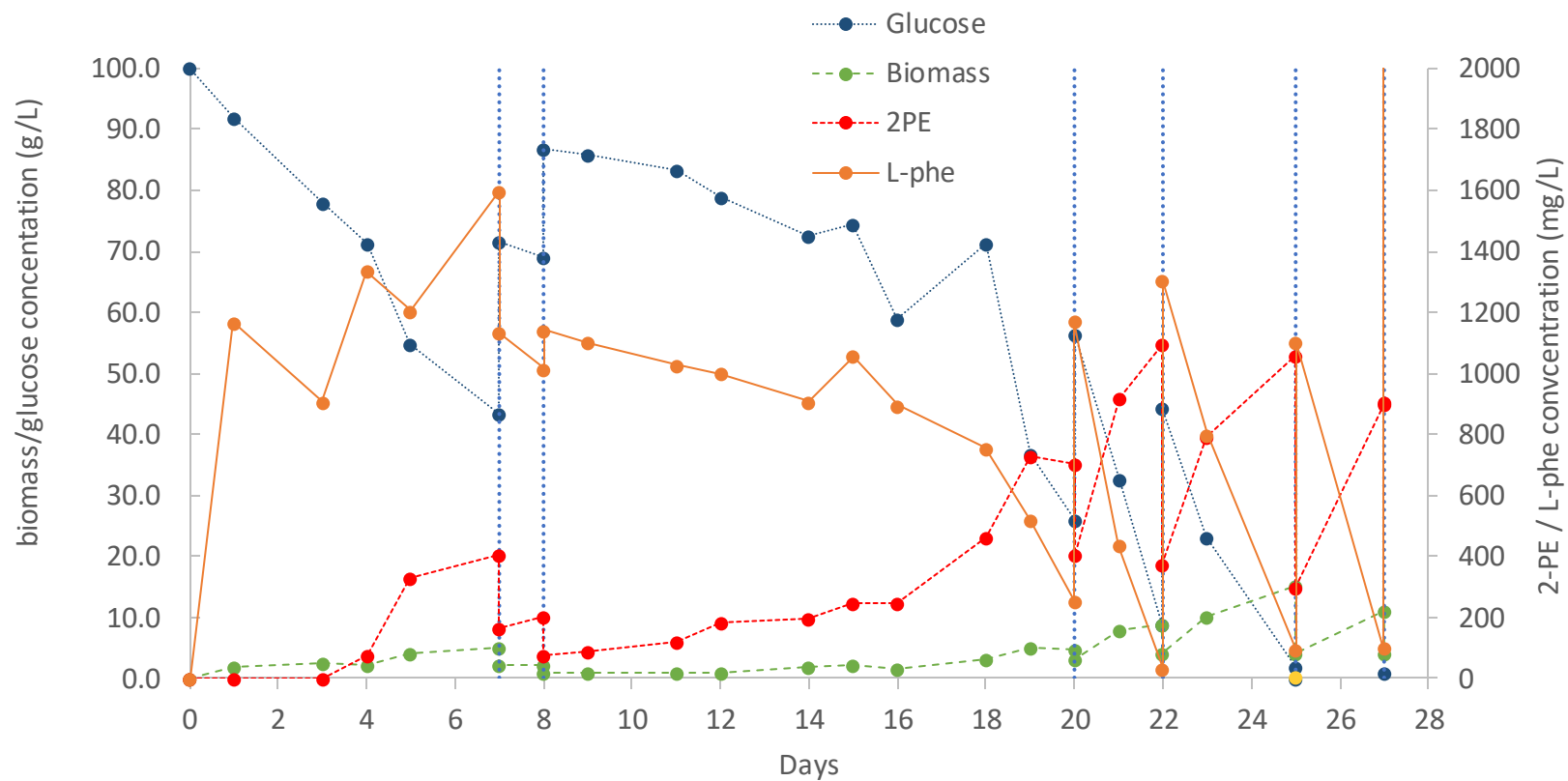


Figure 5.7 2PE production in semi-continuous *M. pulcherrima* bioconversion bioreactor with dilution ratio 0.5. It was cultured in SGJ media with L-phenylalanine 2 g/L controlled pH4 and 20°C with aeration 2L/min. L-phe was estimated roughly from the HPLC-UV at 216 nm without prior modification. (Blue-dotted lines indicated the newly media replaced time)

5.5 Conclusion

In the previous chapter, *M. pulcherrima* was demonstrated to produce 2PE through the conversion of L-phenylalanine via the Ehrlich pathway. In this chapter the process was demonstrated to be effective when scaled up to 2L. Compared to *de novo* 2PE production, 2PE bioconversion can produce 2PE faster and it is not restricted to growth period. Activated carbon was used as the adsorbent as in the bioconversion, it was found to be vital that the 2PE concentration was kept below the inhibitory level of approximately 1500 mg/L.

In the two-phase partitioning bioreactor (TPPB) using activated carbon as an in-situ adsorbent produced 2PE up to 4 g/L working volume, albeit this was slightly lower than observed in the in-situ adsorbent in shake flask fermentation. Glucose is vital to the fermentation and should be available at all time during the process. While the yields from the biosynthesis are high, it should be noted that it is possible substantial conversion of glucose to 2PE is also taking place. Attempts to change the fermentation mode to continuous were unsuccessful, mainly due to contamination of the bioreactor. It was reasoned that the continual removal of 2PE also removes the other antibacterial agents that allow the yeast to operate under non-sterile conditions so effectively.

The optimal conversion mode for the yeast was found to be fermentation in a semi-continuous bioreactor, as 2PE can be rapidly produced but keeping 2PE concentration below the inhibitory concentration. The process yielded 0.5 g 2PE L⁻¹ per L-phe g⁻¹ with the dilution ratio of 0.5. The biomass can be increased by leave the major yeast biomass settle at the bottom before replacing the supernatant with the new media.

In *de novo* 2L bioreactor, the fermentation can yield 2PE 0.7g/ 100g glucose in the cultivation period 5 days. With the estimated cost of glucose, according to Alibaba.com, approximately 420-490 \$/ton, it would be profitable for 2PE production for food grade (\$1000/kg, according to Alibaba.com). In addition, the biomass of the yeast can be considered as a by-product that potentially valuable for lipid production and nitrogen-rich feedstock and fertiliser.

In the calculation based on *de novo* production in 50L bioreactor, 2PE can be produced by batch mode and continuous mode which yields, respectively, 35 g 2PE with the cultivation period 5 days in the batch mode and 5.33 g 2PE/day in the continuous mode with the operation at dilution ratio 1/6.

In the 2PE bioconversion from L-phenylalanine, it might be more attractive mode of production as L-phenylalanine 1 kg (\$3-5/kg, according to Alibaba.com) can be converted to 0.74 g 2PE by *M. pulcherrima* without lost. The maximum production in

batch mode with L-phenylalanine as precursor can yield 13.6 g/L in the batch mode with additional L-phe 20g/L and activated carbon 100g/L. The cost analysis of substrate used in 2PE production from *M. pulcherrima* both from de novo production and L-phenylalanine bioconversion, showing that substrate cost in the production 1 kg (\$1000) would be \$78.6 in de novo production from glucose and \$15.9-18.8 in bioconversion process.

Table 5.1 Estimated cost of substrate in 2PE production (based on 1L Bioreactor) ^(a)

\$		de novo Production	L-phe bioconversion	Unit cost (\$/kg)
Cost	Glucose	0.045	0.045	0.45
	Activated Carbon	-	0.1	1
	L-phenylalanine	-	0.06-0.1	3-5
	Nutrients (approximated)	0.01	0.01	
	Summary (substrate)	0.055	0.22-0.26	-
Products	2-phenylethanol	0.7	13.6	1000

Table 5.2 Estimated cost of substrate in 2PE production (based on 2PE 1 kg) ^(a)

\$		de novo Production	L-phe bioconversion	Unit cost (\$/kg)
Cost	Glucose	64.3	3.3	0.45
	Activated Carbon	-	7.4	1
	L-phenylalanine	-	4.4-7.3	3-5
	Nutrients (approximatly)	14.3	0.73	-
	Summary (substrate)	78.6	15.9-18.8	-
Products	2-phenylethanol	1000	1000	1000

^(a) Noted that the cost was evaluated on only substrate cost where other costs such as operational and construction cost were not included.

The 2PE production from *M. pulcherrima*, either the de novo culture from glucose or bioconversion from L-phenylalanine, has demonstrated that far better than extraction from natural roses or other flowers. It is easier to cultured and to control the production environment in bioreactor, less time consuming and minimise land use. It is also more cost effective in production as well.

5.6 References

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Chapter 6

Materials and Methodology

6.1 Materials and yeast

All chemicals were mostly supplied by Sigma Aldrich as analytical grade or equivalent unless stated otherwise. *M. pulcherrima* was obtained from National Yeast Culture Collection (Norfolk, UK) and stored on sterile YPD agar plate at 4°C. The stock culture was re-plated every two months to ensure the culture viability. Waste glycerol was provided by Croda Europe and used as received.

6.1.1 Inoculation

M. pulcherrima was inoculated from the yeast stock plate in YMS media and incubated at 20°C and shaking at 150 rpm for 48 hours.

6.1.2 Media

6.1.2.1 Synthetic grape juice (SGJ) media and its modified formulas

Synthetic grape juice (SGJ) media was adopted from the literature [166] to replicate grape must in wine fermentation. The media concentrations are given below

Table 6.1 Media Formula in *M.pulcherrima* study

Formula (g/L)	SGJ Media	SGJ (glucose) Media	MSGJ (C/N 100:1) media	XGJ (xylose) media	MXGJ (xylose) media	Glycerol media
glucose (+)	70	100	70			
xylose				70	70	
glycerol						70
fructose	30		30	30	30	30
tartaric acid	7	7	7	7	7	7
malic acid	10	10	10	10	10	10
(NH ₄) ₂ HPO ₄	0.67	0.67	3.75	0.67	3.75	0.67
KH ₂ PO ₄	0.67	0.67	0.67	0.67	0.67	0.67
MgSO ₄ *7H ₂ O	1.5	1.5	1.5	1.5	1.5	1.5
NaCl	0.15	0.15	0.15	0.15	0.15	0.15
FeSO ₄ * 7H ₂ O	0.021	0.021	0.021	0.021	0.021	0.021
ZnSO ₄ *7H ₂ O	0.0075	0.0075	0.0075	0.0075	0.0075	0.0075
CaCl ₂	0.15	0.15	0.15	0.15	0.15	0.15

6.1.2.2 YMS medium

YMS medium used in this report was composed of 30 g/L yeast extract, 5 g/L mannitol and 5g/L sorbose and used in all *M. pulcherrima* inoculations unless otherwise stated

6.1.2.3 YPD medium

YPD medium was composed of 10 g/L yeast extract, 20 g/L peptone and 20 g/L glucose and used in agar plate culture in reculturing *M. pulcherrima* stock.

6.1.3 Sterilisation

All media and glassware in *M. pulcherrima* culture was sterilised at 121°C for 15 min in an autoclave prior to use

6.2 Chapter 2

6.2.1 Media selection

M. pulcherrima was inoculated and cultured in 125mL Erlenmeyer flasks with 25 mL working volume with SGJ and MSGJ media in triplicate. The cultures were incubated on a shaker at 150 rpm at 20°C. Samples were collected daily from triplicate flasks to analyse biomass and a selected sample was analysed by HPLC analysis.

In depletion study of nitrogen, phosphorus and acids, the studied compounds were reduced to 1/10 of regular formula while the other compounds were unchanged. In the study of phosphate depletion, $(\text{NH}_4)_2\text{SO}_4$ was used instead of $(\text{NH}_4)_2\text{HPO}_3$. The study were done in triplicate in the experiment and analysis similar to the former study.

6.2.2 Assessment of fermentation products formed in the fermentation

Samples were from 6.2.1 were selected to analyse by HPLC-RI analysis. The unknown compounds were collected from the HPLC outlet at each retention time and then analysed by Mass Spectroscopy technique. Arabitol was confirmed using arabitol solution 1 g/L (diluted 20 times) compared to ribitol and xylitol solutions.

6.2.3 Optimisation of *M. pulcherrima* fermentation

In pH optimisation, *M. pulcherrima* was inoculated and cultured in 125mL Erlenmeyer flasks with 25 mL working volume with SGJ with modified pH at 2, 3, 4 and 5 with tartaric and malic acid as buffer and internal standard. The culture were cultured in triplicate. The cultures were incubated on a shaker at 150 rpm at 20°C. Samples were collected daily from triplicate flasks to analyse biomass and a selected sample was analysed by HPLC-RI and HPLC-UV analysis.

In temperature optimisation, the similar experiment and the analysis to the pH optimisation were conducted with SGJ media modified to pH4. The cultures were incubated at 15, 20 and 28 °C.

In nitrogen/carbon ratio study, *M. pulcherrima* was cultured in SGJ media with $\text{NH}_4(\text{HPO}_3)_2$ as the sole nitrogen source at different concentrations, (0.1, 0.2, 0.5 and 1

g N/ 100 g carbon). The SGJ media contained glucose at 100 g/L as substrates. The end products were analysed at the end of fermentation at 7 days. The conditions and analysis were similar to the previous study.

The synergistic 2-phenylethanol and ethanol toxicity tests were carried out using 96-microplates with 2PE, ethanol and YMS media mixture at the preferred concentrations. In each of the wells, *M. pulcherrima* inoculum (50 hrs old, 3 μ L) was added to MSGJ media 195 μ L and incubated for 7 days at 20°C, with shaking at 180 rpm. The inhibition to *M. pulcherrima* growth was determined by OD at 600 nm by the microplate reader (Versamax, Molecular devices UK).

6.2.4 Effect of strain variation

M. pulcherrima 4 strains was mutated in laboratory from the courtesy of Dr. Daniel Henk in Department of Biology, University of Bath. The culture, condition and the analysis are identical to the previous study.

6.2.5 Alternative substrates

The XGJ and MXGJ media were used in the xylose study, as the formula were shown in table 6.1. The culture, condition and the analysis are identical to the previous study.

The glycerol media was used in the glycerol study, as the formula were shown in table 6.1. Glycerol was the waste glycerol from biodiesel production from Croda Company (UK). The culture, condition and the analysis are identical to the previous study.

6.3 Chapter 3

6.3.1 Effect of aeration in 2L bioreactor.

Experiments in bioreactors were conducted in 2L Fermac 320 Bioreactor Fermenter (Electrolab Biolab Ltd.). Temperature, pH and dissolved oxygen (DO) were controlled through the controller system using probes and could be kept constant, if needs be. The threshold for automatic control was $\text{pH} \pm 0.05$, temperature ± 0.1 °C and DO ± 1 unit. The bioreactor jar was sterilised prior to fermentation through autoclaving at 121 °C, 15 minutes. The different aeration at 0.5, 1 and 2 L/min were applied in separated experiment.

6.3.2 Semi-continuous *de novo* 2PE production

In the semi-continuous bioreactor experiment, the culture was started similar to the batch fermentation in the 2L bioreactor. When it reached growth phase, the semi-continuous fermentation was initiated by taking the supernatant out and adding new medium in the same volume. The output volume was according to the parameter controls which are

dilution ratio and hydraulic retention time (HRT). It could be calculated given dilution ratio and HRT are defined as

$$Dilution\ ratio(day^{-1}) = \frac{\dot{V}}{V_0} (day^{-1})$$

$$Hydraulic\ Retention\ Time\ (day) = \frac{1}{Dilution\ ratio} = \frac{V_0}{\dot{V}}$$

Therefore,

$$\dot{V} = V_0 \times Dilution\ ratio = \frac{V_0}{HRT}$$

Where \dot{V} = volume taken out per day ($L \cdot day^{-1}$)

V_0 = working volume of bioreactor (L)

In the continuous bioreactor experiment, the input and output flow was taken out by the peristaltic pump. The input flow rate \dot{V} was calculated by similarly to the semi-continuous and controlled by the feeding peristaltic pump. The overflow output was taken out by foaming peristaltic pump at the top level of the fermenter.

The ex situ adsorption column used plain chromatography column with I.D. 26mm x 457 mm, pack with 50 g activated carbon, approximately bulk column volume 100 mL and cross-sectional area 5.3 sq.cm.

The cross-flow membrane unit in Chapter 5 used the MiniKros® Sampler Filter Modules supplied from Spectrum labs, Co. Ltd. The pore size is 750 kD and fiber ID 1.0 mm. The column length is 20 cm with surface area 490 sq.cm.

6.4 Chapter 4

6.4.1 2PE production by biosynthesis from L-phenylalanine

M.pulcherrima was culture in the similar ways as in described 6.2.3 with L-phenylalanine 10, 20 and 30 g/L was added in SGJ media without other nitrogen compound. The culture, condition and the analysis are identical to the previous study.

6.4.2 *In situ* liquid-liquid adsorption in 2PE biosynthesis production

Two solvents were selected for screening liquid-liquid in-situ 2PE extraction in the biosynthesis; oleyl alcohol (OA) and dodecane (DDC). Firstly, the toxicity of both solvents to *M. pulcherrima* was studied in 25mL culture in 125 mL shaking flasks, incubating at 20 °C for 15 days. The culture was conducted in SGJ media with added L-phenylalanine 2 g/L as a sole nitrogen source. The solvent toxicity was determined by the yeast cell mass.

6.4.3 *In situ* 2PE adsorption in activated carbon and ZSM5

In comparison 2PE adsorption capacity of solid adsorbents (powdered activated carbon and ZSM5, 0.5 g of adsorbent was added in 10 mL aqueous 2PE solution of concentration 1000mg 2PE/L. The mixture was agitated at 20°C for 3 days. The remained 2PE concentration in the aqueous phase was analysed by HPLC-UV.

6.4.4 Langmuir adsorption capacity (Figure 4.8)

Langmuir adsorption characteristic and maximum capacity was studied by using activated carbon 0.10 g in aqueous 2PE concentration from 10, 20, 40, 60, 80 and 100 µL in 10 mL distilled water.

6.4.5 2PE solvent leachability study (Figure 4.10)

In study of 2PE solvent leachability, 2PE adsorption equilibrium in solvents were conducted. PAC 0.1 g and 2PE 10.0 µL were added to distilled water 10 mL and shaken at 150 rpm, 20°C overnight. Residual 2PE in aqueous phase was determined by HPLC-UV.

6.5 Chapter 5

6.5.1 2PE adsorption on different size of activated carbon

In the study of 2PE adsorption on different size of activated carbon, the adsorbent was used in 4 different sizes, powdered, granular Draco 20-40 mesh, granular Draco 4-12 mesh, which are all supplied by Sigma Aldrich and granular commercial activated carbon Fluval Carbon, purchased from Amazon UK, typically used in household water purification equipment. The adsorption study used an initial 2PE aqueous solution of 1000 mg/L and added activated carbon 1g / 50 mL solution in 125 mL Erlenmeyer flasks,

shaking at 20°C and 180 rpm. The experiments were done in triplicate for each size. The samples were collected from the aqueous phase after 5 min, 10 min and 2 hours and analysed for 2PE.

6.5.2 2PE fermentation in TPPB with activated carbon as an *in situ* extractant and controlled in batch mode bioreactor

M. pulcherrima was cultured in the TPPB with activated carbon as an *in-situ* extractant and controlled in batch mode. The media for bioconversion was SGJ media with the addition of 20g/L of L-phenylalanine. Activated carbon (GAC2040, 20-40 mesh) was sterilised and added directly in the bioreactor in steps of 20 g or 50 g when 2PE in the aqueous phase was over 500 mg/L. The purpose of stepped adding GAC was to trace 2PE concentration in the bioreactor which could not be traced if GAC was overloaded and adsorb all the traceable 2PE produced. The threshold of 500 mg/L was selected as the requirement for adding new activated carbon. The maximum activated carbon was 150 g.

6.5.3 Attempts on 2PE fermentation in TPPB with activated carbon as an *in situ* extractant and controlled in continuous mode bioreactor

The fermentation in bioreactor was started as batch mode similar to the previous study and the continuous mode was started since day 3. To conduct continuous mode, the input feed was fed directly into the bioreactor and the output was flowed through the adsorption column with GAC0240 (20-40 mesh) as the adsorbent. 2PE was removed by the absorbent and the rest of the stream sent to waste. When the adsorption in the column was at its limit, methanol was used as the solvent to leach 2PE from the column. 2PE was easily recovered from the methanol using simple evaporation of the solvent. The samples were collected in bioreactor and the filtrate from the adsorption column. /-

6.6 Analysis

6.6.1 Yeast biomass

M. pulcherrima biomass during fermentation was determined by optical density at 600 nm by an UV/Vis spectrometer. The samples were diluted to make maximum optical density less than 1.0, usually with 20 times dilution. The OD was converted to biomass by previously studied correlation.

6.6.2 2-phenylethanol

2-phenylethanol was determined on a HPLC system (Shimadzu 10AVP HPLC system with LC-10AD pump, auto injector (SIL-10AD) with Hypersil C-18 reverse phase column (Thermo Inc.) and UV detector at 216nm. The LC eluent was acetonitrile/water 60:40,

flowing at 0.8 mL/min. Samples were diluted in range of 0-35 mg/L and filtered through 0.22 µm filter prior to analysis.

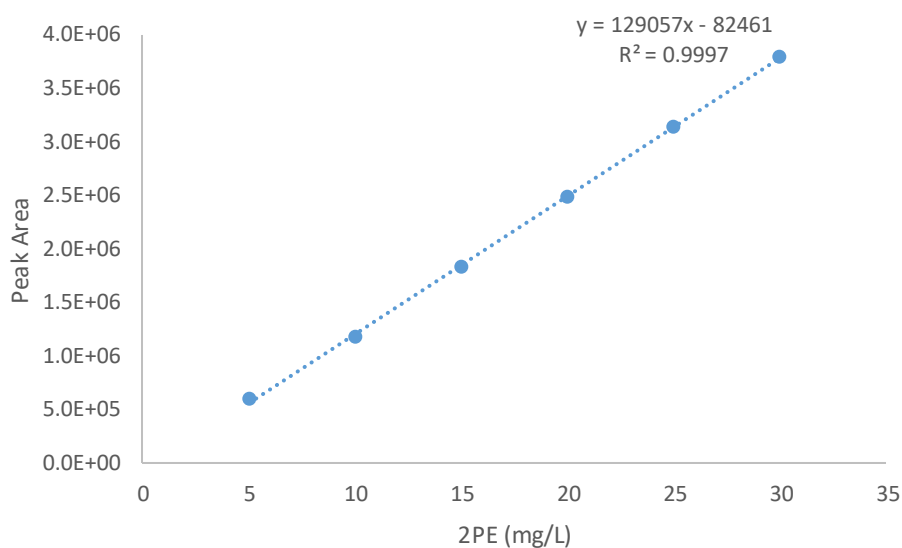


Figure 6.1 Calibration curves for 2PE, determined by HPLC (Shimadzu 10AVP HPLC system) with UV detector and C18 column (Thermo Scientific)

6.6.3 Sugars, Glycerol, Organic acids and Ethanol

All sugars, organic acids and ethanol were determined by HPLC system (identical to 2-phenylethanol analysis) with Aminex HPX-87H column (60°C) and Refractive Index Detector (40°C). The LC eluent was H₂SO₄ 5 mM flowing at 0.6 mL/min. Samples were diluted in range of 0-5 g/L and filtered through 0.22 µm filter prior to analysis.

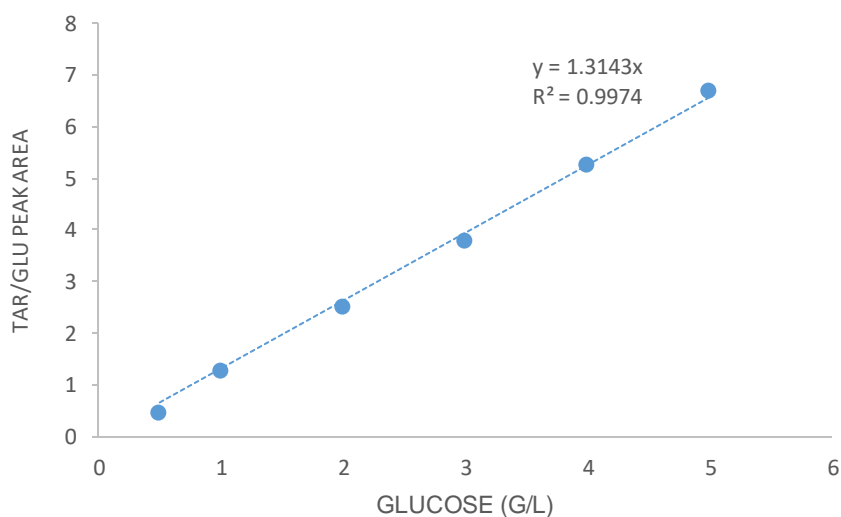


Figure 6.2 Calibration curves for glucose, analysed by HPLC (Shimadzu 10 AVP HPLC Sytem) with Aminex HPX87H column and RI detector. The concentration is determined with tartaric acid 7 g/L as an internal standard.

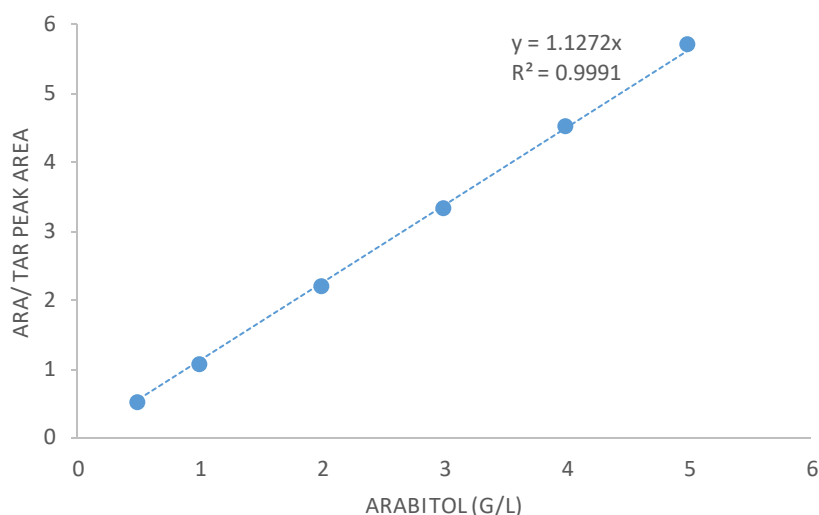


Figure 6.3 Calibration curves for arabitol. analysed by HPLC (Shimadzu 10 AVP HPLC Syntem) with Aminex HPX87H column and RI detector. The concentration is determined with tartaric acid 7 g/L as an internal standard.

6.6.4 Gas Chromatography and Mass Spectroscopy

The preliminary GC-MS analysis of volatile compounds production from fermentation was carried out using an Agilent 7890A Gas Chromatograph equipped with a capillary column (60m × 0.250mm internal diameter) coated with DB-23 ([50%-cyanopropyl]-methylpolysiloxane) stationary phase (0.25µm film thickness) and a He mobile phase (flow rate: 1.2ml/min) coupled with an Agilent 5975C inert MSD with Triple Axis Detector.

The sample was prepared for analysis by taking 50 ml of the supernatant from the fermentation broth. The sample was centrifuged to remove the yeast cells and the metabolites were extracted with 5ml of dichloromethane (x2) The dichloromethane was removed using a rotary evaporator at 40 °C until 500uL remained. The sample was diluted with 1,4 dioxane (2 mL) and 4-octanol was used as an internal standard.

In confirmation of arabitol production from the fermentation, the unknown compound in fermentation supernatant was separated by HPLC with Aminex HPX-87H column and collected at preferred retention time. The HPLC condition is identical to sugars and organic acids analysis. The collected sample was then injected to an Electrospray Quadrupole Time-of-Flight (ESI-QTOF) mass spectrometer to determine the atomic weight of unknown compounds.

6.7 References

1. Bejaoui, H., et al., *Ochratoxin A removal in synthetic and natural grape juices by selected oenological Saccharomyces strains*. Journal of Applied Microbiology, 2004. **97**(5): p. 1038-1044.

Chapter 7

Conclusions and Future Work

7.1 Conclusions

2-phenylethanol (2PE) is a higher molecular weight alcohol with a rose-like aroma. It is valuable aromatic compound in the fragrance, cosmetic, food and beverage industry. Previously, it was produced mainly from chemical processes from petroleum-based aromatic precursors such as benzene or styrene oxide. The major drawback of chemical production of 2-PE is the separation, which is tedious and difficult to purify 2PE and as such has various by-products that make it unsuitable for use in the food and beverage industry. The biological approach, while higher cost, uses less extreme conditions and produces a more pure product safe to use in the food sector. The biological production of 2PE can be done via fermentation with microorganisms, mainly yeasts. The fermentation can be conveyed with 2 different ways, the first is *de novo* conversion from glucose and the second is the bioconversion of L-phenylalanine via Ehrlich pathway. *De novo* production from glucose provides the advantage of using glucose as substrate which is relatively cheaper but with the drawback of the less 2PE, generally less than 0.5 g/L. The bioconversion from L-phenylalanine would give higher 2PE concentration in the fermentation broth but it would require the expensive amino acid L-phenylalanine as a precursor.

In this thesis, *M. pulcherrima* was demonstrated to be an excellent microorganism platform for 2PE production both in *de novo* conversion from glucose and bioconversion from L-phenylalanine with an efficient percent conversion. It was shown to be the best *de novo* 2PE producer in the literature producing more than 1 g/L 2PE from 100 g/L sugar media in the shake flasks experiment. 2PE production was reduced to 800 mg/L in a 2L bioreactor, however, this was still in excess of any reported *de novo* 2PE production. The production of 2PE is mostly complete in the growth phase in the first 3-5 days. *De novo* 2PE production depends highly on the carbon flux through the pentose phosphate pathway and the shikimate pathway which, in yeast, seemingly, are restricted in the growth period in the limited nitrogen condition. Such condition triggers the carbon flux through the shikimate pathway to create aromatic amino acids such as phenylalanine, tyrosine and tryptophan.

L-phenylalanine is then converted to 2PE through the Ehrlich pathway. It seems that, unlike bacteria, most yeasts possess keto decarboxylase (KDC) and alcohol dehydrogenase (ADH) which are the important enzymes that convert phenylpyruvate to phenyl aldehyde and phenyl aldehyde to 2-phenylethanol, respectively. It is possible that *M. pulcherrima* produces mostly L-phenylalanine among other aromatic amino acids because the others were barely detected in this study under any of the conditions examined.

In 2PE biosynthesis from L-phenylalanine, the process is gone through Ehrlich pathway which is capable in most yeasts, albeit difference in end conversion success are expected, due to competitive side reaction of L-phenylalanine catabolism in some yeasts. From the academic literature, *K. marxianus* can convert L-phenylalanine to 2PE the most efficiently. In this study, however, *M. pulcherrima* was shown to be as capable with similar titres produced in the L-phenylalanine bioconversion. *M. pulcherrima* is therefore an excellent platform for production of 2PE from either glucose or L-phenylalanine.

Seemingly, there are barely barriers for *M. pulcherrima* to synthesise 2PE from L-phenylalanine as long as 2PE concentration in the broth is kept under the inhibition threshold which is approximately 1.5-1.7 g/L for *M. pulcherrima*. This study also found that the threshold can be lower to 1.2 g/L synergistically with the addition of 4%v/v ethanol.

It was thought previously that the action of biological control from *M. pulcherrima* was dependent on the iron depletion mechanism, presumably by the pulcherrimic acid which is a chelating agent, forming a purple red pigment. But it might be possible that 2PE and other esters and alcohols play some part in the microbial control activity as well.

In-situ extraction in 2PE biosynthesis fermentation is vital to control 2PE concentration and not to exceed the inhibitory level to the yeast. The extractants could be non-toxic liquid solvent or solid adsorbents/ion exchangers. It was found in this study that oleyl alcohol was a good candidate for in-situ liquid solvent because of its non-toxicity to microorganisms, its less likely to form emulsion and separated clearly from the aqueous phase and high 2PE solubility. However, excessive liquid solvent addition leads to reduced oxygen transfer and, consequently, reduced yeast performance. This study suggested the added oleyl alcohol should not exceed 20% of the working volume. Its disadvantage though is 2PE separation from the solvent itself. The alternative in-situ solid extraction could be more attractive as it provides various choice of suitable adsorbents and the 2PE separation is not technically difficult.

Activated carbon was demonstrated to be an excellent 2PE adsorbent. Its maximum Langmuir 2PE adsorption capacity was found to be approximately 800 mg 2PE/g activated carbon. It is inexpensive and the inert non-toxic nature to the yeast are also an advantage. The adsorption rate and capacity seemed to be affected by activated carbon size. The powder activated carbon is the fastest adsorbent and has the highest capacity, but the fine particle size presented technical difficulties in the separation and is probably impractical for use in adsorption and desorption columns. The coarse granular activated carbon with size as large as 4-12 mesh, similar to the commercial granular activated

carbon, was perfect for use in the column, but higher loadings would be needed to compensate with the lower adsorption rate and capacity. Activated carbon size 20-40 mesh seems to be the best compromise option between both and did the extraction efficiently in this study in terms of adsorption capacity and satisfying adsorption rate.

The performance of in-situ activated carbon in the fermentation may have been affected by the conditions used in the fermentation. The optimal growth and 2PE production of *M. pulcherrima* was at pH 4, but this pH reduces the adsorption capacity of activated carbon. The effect of pH to adsorption is well known. This should be concerned in the in-situ 2PE removal by adsorption and the performance at the working pH should be studied prior to bioreactor design process. It is also clear that biomass clogging in the micropores of the adsorbent can reduce the adsorption capacity and should be recognised in the design process as well.

Several fermentation operation regimes were conducted in this study, including batch, semi-continuous and continuous with in-situ and ex-situ adsorption in the 2PE biosynthesis fermentation. Batch experiment was simple to operate but needed time in the lag phase and early log growth phase which is a non-productive period. The continuous fermentation with ex-situ activated carbon adsorption promised to be a flexible operation which can include 2PE leaching in the process but it was found to be susceptible to contamination due to the microbial control agents seemingly adsorbed onto activated carbon which led to the vulnerability of the culture. From this research it seems clear that semi-continuous processing may be the best way to control biomass and the rate of 2PE conversion.

In general, yeasts seem to possess the ability to convert amino acids to higher alcohols through Ehrlich pathway very well, yielding products such as 2PE. Though, it lacks the ability to convert carbohydrate to aromatic amino acids which can found in plants and bacteria through the shikimate pathway, this pathway can only be triggered by limitation nitrogen in the growth phase in yeast fermentation. While *M. pulcherrima* seemed to channel carbon flux through shikimate pathway better than other yeasts, it still incomparable to bacteria. However, bacteria cannot be used in 2PE production as well as they lack KDC and ADH enzymes which is crucial to higher alcohol conversion in Ehrlich pathway. This suggests that in higher alcohols production such as 2PE, it could benefit from combining yeasts and bacteria abilities together. It could be co-fermentation or genetic modification of the microorganisms in the future study.

Another attractive by-product by *M. pulcherrima* fermentation is D-arabitol, which the yeast assumingly produces along with glycerol as a response to the high osmotic stress. In some conditions in this study, *M. pulcherrima* can make D-arabitol up to 20 g/L.

However, the condition of D-arabitol production may be less inconsistent and not well understood enough. Arabitol could be important in future as another building block chemical as a platform for bio-products. Its current production is still low, but recently research has been published to use a wider range of sugar-derived compound that can produced through metabolic pathway in microorganisms. With a better understanding, arabitol production could be a significant biochemical building block in the future.

M. pulcherrima has been demonstrated that it would be a great platform for 2PE production either de novo glucose conversion or L-phenylalanine bioconversion. It also has recently raised interest in lipid production which its lipid resembles palm oil quality and its ability to grown in the less sterile conditions, it promises to be a great platform of lipid production in the next generation. The condition of lipid production is similar to the condition of 2PE production which is generally low nitrogen with high carbohydrate available. Such conditions would trigger the aromatic amino acids production which leads to 2PE production through Shikimate and Ehrlich pathway and also induce the yeast to produce high carbon compound such as lipid due to nitrogen limitation. This would suggest *M. pulcherrima* can be used as co-fermentation for both lipid and valuable 2PE as a by-products providing the platform for a working biorefinery

7.2 Future work

It seems clear from this study that 2PE can be produced through microbial fermentation and shows good potential for scale up. If it is possible to produce on the larger scale the increased supply to the market would eventually reduce the production costs and increase the use of 2PE to a wider range of bulk chemicals, which are now limited to the non-food products such as fragrance and perfumes. 2PE from natural origin is allowed to food and beverage industry. Therefore, 2PE from fermentation is likely to increase use of 2PE particularly in food, beverage and cosmetic industry. However, the future application of 2PE may be in the another promising field: fuels. The use of branched-chain higher alcohols, including 2PE, in fuels was proposed by Atsumi *et al.* as they possessed higher energy density and were less likely to collect water in storage. Though its current limitation is the production volume capability.

2PE can be synthesised via the *de novo* synthesis from carbohydrates or by bioconversion from L-phenylalanine by several yeasts. In the *de novo* 2PE synthesis, *M. pulcherrima* appears to be the highest 2PE producer, however, the production is less productive in the biosynthesis from L-phenylalanine. Further work needs to be invested in the scale up of the *de novo* system as it presents the advantage of non-toxic condition to the fermentation and, therefore, does not require sophisticated two-phase system and precursor. Though the fermentation is rather slow and takes a relatively long time on the lab scale, this could be speeded up through strain selection or optimal reactor condition. Semi-continuous fermentation may also be an interesting mode of fermentation because of its balance between less time for growth period and less lag phase and growth time, though this would need to be demonstrated on a larger scale.

In 2PE biosynthesis from L-phenylalanine, *M. pulcherrima* presents an excellent choice for 2PE biosynthesis because the 2PE toxicity threshold to the yeast is high and L-phenylalanine is mostly converted to 2PE with minimal loss to side-reactions. There also seems some level of *de novo* synthesis as well. The present difficulty for 2PE biosynthesis would be the choice of extractants either in the in-situ or ex-situ fermentation. The in-situ extractive fermentation would be easier to operate but there would be limitation of 2PE extraction capacity, while the ex situ extractive fermentation would be more complex operation and more prone to contamination. Again these systems need to be tested on a larger scale system. Activated carbon was demonstrated to be an excellent choice for 2PE adsorption with huge adsorption capacity but, technically, it is more susceptible to contamination as its natural biocontrol agents might be adsorbed onto the carbon and so will not be effective on a larger scale

However, *de novo* 2PE production with 2 fermentation units, for L-phenylalanine biosynthesis by bacteria and 2PE biosynthesis from L-phenylalanine by yeast, could be an interesting approach to 2PE production on the large scale. This is because the pathway of *de novo* 2PE production involves two connected pathways which are shikimate pathway, which yield L-phenylalanine, and Ehrlich pathway, which convert L-phenylalanine to 2PE. Shikimate pathway is commonly found in plants and bacteria but, in yeast, it is limited to the growth condition under limited nitrogen. For example, it was reported to be able to produce L-phenylalanine from glycerol with high conversion yield (0.58 g/g) from *E. coli*. Ehrlich pathway, however, is found in several yeasts and can yield high percent conversion. The two separated fermentations could then benefit than solely yeast fermentation which is more restricted.

The other alternative choice could be the genetic modification of the microorganism to possess the ability of metabolise through both shikimate pathway and Ehrlich pathway. One another advantage of *M. pulcherrima* is that it is oleaginous yeast which can be used as lipid producer and also produce 2PE. The production of both compounds shares the similar condition with is high carbohydrate and low nitrogen. In the increasing significance of sustainable lipid production, *M. pulcherrima* could potentially produce lipid and also generate 2PE as a valuable by-product reducing the costs substantially of both products.